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     FILE 'USPATFULL' ENTERED AT 07:33:54 ON 16 APR 2007
                 E DORR ALEXANDER P/IN
L1             2 S E2 OR E3
                 E OTT MELANIE/IN
L2             5 S E3 OR E4
L3             4 S L2 NOT L1
                 E VERDIN ERIC/IN
L4             5 S E3
L5             2 S L4 NOT (L1 OR L2)

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                 E OTT M/IN
L7            81 S E3
L8             4 S L7 AND TAT
L9             3 S L8 NOT L6
                 E VERDIN E/IN
L10            8 S E3
L11            5 S L10 NOT (L6 OR L8)

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                 E DORR A P/AU
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                 E OTT MELANIE/AU
L13           17 S E3
L14           12 S L13 NOT L12
                 E VERDIN ERIC/AU
L15           37 S E3
L16           30 S L15 NOT (L12 OR L13)
L17            2 S L16 AND TAT

     FILE 'USPATFULL' ENTERED AT 07:50:36 ON 16 APR 2007
L18        51102 S (HIV OR HUMAN IMMUNODEFICIENCY VIRUS)
L19        10928 S L18 AND TAT
L20         2776 S L19 AND ACETYLAT?
L21          125 S L20 AND TAT/CLM
L22            8 S L21 AND ACETYLAT?/CLM

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L24          844 S L23 AND TAT
L25           11 S L24 AND ACETYLAT?
L26            8 S L25 AND TAT/AB
L27            6 S L26 AND ACETYLAT?/AB

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L28       175185 S (HIV OR HUMAN IMMUNODEFICIENCY VIRUS)
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L30           60 S L29 AND ACETYLAT?
L31            3 S L30 AND ANTIBOD?
L32           57 S L30 NOT L31
L33           43 S L32 AND PY<2005
L34           40 S L33 NOT (L12 OR L13 OR L15)

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L37          196 S L36 AND ANTIBOD?/CLM
L38           46 S L37 AND (ANTI-TAT)
L39           33 S L38 AND AY<2004
L40           10 S L39 AND (TAT/TI OR ANTIBOD?/TI)

     FILE 'MEDLINE' ENTERED AT 08:58:21 ON 16 APR 2007

     FILE 'USPATFULL' ENTERED AT 08:58:29 ON 16 APR 2007
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L42          289 S L41 AND (CYSTEINE-MODIFICATION OR CYSTEINE ADDITION OR MODIFI
L43          235 S L42 AND CONJUGAT?
L44           75 S L43 AND POLYPEPTIDE?/CLM
L45           24 S L44 AND CYSTEINE?/CLM
L46           14 S L45 AND (CARRIER/CLM OR CONJUGATE?/CLM)
L47           18 S L45 AND AY<2004
L48           11 S L46 AND AY<2004

     FILE 'MEDLINE' ENTERED AT 09:04:17 ON 16 APR 2007
L49       485570 S (PEPTIDE? OR POLYPEPTIDE?)
L50          145 S L49 AND (C-TERMINAL CYSTEINE)
L51           23 S L50 AND (IMMUNOGEN? OR VACCIN? OR CONJUGAT? OR CARRIER?)
L52           13 S L51 AND PY<2004
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acids, from about 35 amino acids to about 40 amino acids, from about 40 amino acids to about 45 amino acids, from about 45 amino acids to about 50 amino acids, from about 50 amino acids to about 55 amino acids, from about 55 amino acids to about 60 amino acids, from about 60 amino acids to about 65 amino acids, from about 65 amino acids to about 70 amino acids, from about 70 amino acids to about 75 amino acids, from about 75 amino acids to about 80 amino acids, from about 80 amino acids to about 90 amino acids, or from about 90 amino acids to about 100 amino acids in length, up to the full-length Tat polypeptide, of any one of the amino acid sequence set forth in Figures 5A and 5B.

[0042] In some embodiments, a subject acetylated Tat polypeptide comprises the following consensus sequence (where amino acid sequences are provided from amino-terminus (N-terminus) to carboxyl-terminus (C-terminus): Lys-(Ala or Gly)-Leu-Gly-Ile-Ser-Tyr-Gly-Arg-Lys-Lys-Arg-(Arg or Lys)-(Gln or His)-Arg-Arg-(Arg or Gly or Lys or Ser)-(Pro or Ala or Thr)-(Gln or Pro or Thr) (SEQ ID NO:01), wherein one or more of the lysines is acetylated. In some embodiments, the lysine corresponding to Lys-50 in the full-length protein is acetylated (e.g., the underlined Lys in SEQ ID NO:01).

[0043] In a particular embodiment, a subject acetylated Tat polypeptide includes the amino acid sequence Ser-Tyr-Gly-Arg-AcLys-Lys-Lys-Arg-Arg-Gln-Arg (SEQ ID NO:02).

[0044] Exemplary, non-limiting acetylated Tat polypeptides include those shown below (where amino acid sequences are provided from amino-terminus (N-terminus) to carboxyl-terminus (C-terminus)):

[0045] Ser-Tyr-Gly-Arg-acetylated Lys-Lys-Arg-Arg-Gln-Arg-Cys (SEQ ID NO:03);

[0046] Ser-His-Gly-Arg-acetylated Lys-Lys-Arg-Arg-Gln-Arg-Cys (SEQ ID NO:04);

[0047] Lys-Gly-Leu-Gly-Ile-Ser-Tyr-Gly-Arg-acetylated Lys-Lys-Arg-Arg-Gln-Arg-Arg-Arg-Thr-Pro-Gln (SEQ ID NO:05);

[0048] Lys-Ala-Leu-Gly-Ile-Ser-Tyr-Gly-Arg-acetylated Lys-Lys-Arg-Arg-Gln-Arg-Arg-Arg-Thr-Ser-Gln (SEQ ID NO:06);

[0049] Lys-Ala-Leu-Gly-Ile-Ser-Tyr-Gly-Arg-acetylated Lys-Lys-Arg-Arg-Gln-Arg-Arg-Arg-Thr-Ala-Gln (SEQ ID NO:07);

[0050] Lys-Gly-Leu-Gly-Ile-Ser-His-Gly-Arg-acetylated Lys-Lys-Arg-Arg-Gln-Arg-Arg-Arg-Thr-Pro-Pro (SEQ ID NO:08);

[0051] Lys-Gly-Leu-Gly-Ile-Ser-Tyr-Gly-Arg-acetylated Lys-Lys-Arg-Arg-Gln-Arg-Arg-Arg-Ala-Ala-Gln (SEQ ID NO:09);

[0052] Lys-Gly-Leu-Gly-Ile-Ser-Tyr-Gly-Arg-acetylated Lys-Lys-Arg-Arg-Gln-Arg-Arg-Arg-Ala-Pro-Gln (SEQ ID NO:10);

[0053] Lys-Gly-Leu-Gly-Ile-Ser-Tyr-Gly-Arg-acetylated Lys-Lys-Arg-Arg-Gln-Arg-Arg-Arg-Ser-Pro-Gln (SEQ ID NO:11);

[0054] Lys-Gly-Leu-Gly-Ile-Ser-Tyr-Gly-Arg-acetylated Lys-Lys-Arg-Arg-Gln-Arg-Arg-Arg-Pro-Pro-Gln (SEQ ID NO:12);

[0055] Lys-Gly-Leu-Gly-Ile-Ser-Tyr-Gly-Arg-acetylated Lys-Lys-Arg-Arg-Gln-Arg-Arg-Arg-Thr-His-Gln (SEQ ID NO:13);

[0056] Gly-Leu-Gly-Ile-Ser-Tyr-Gly-Arg-acetylated Lys-Lys-Arg-Arg-Gln-Arg-Arg-Arg-Thr-Pro- (SEQ ID NO:14);

[0057] Ala-Leu-Gly-Ile-Ser-Tyr-Gly-Arg-acetylated Lys-Lys-Arg-Arg-Gln-Arg-Arg-Arg-Thr-Ser (SEQ ID NO:15);

[0058] Ala-Leu-Gly-Ile-Ser-Tyr-Gly-Arg-acetylated Lys-Lys-Arg-Arg-Gln-Arg-Arg-Arg-Thr-Ala (SEQ ID NO:16);

[0059] Gly-Leu-Gly-Ile-Ser-His-Gly-Arg-acetylated Lys-Lys-Arg-Arg-Gln-Arg-Arg-Arg-Thr-Pro (SEQ ID NO:17);

[0060] Gly-Leu-Gly-Ile-Ser-Tyr-Gly-Arg-acetylated Lys-Lys-Arg-Arg-Gln-Arg-Arg-Arg-Ala-Ala (SEQ ID NO:18);

[0061] Gly-Leu-Gly-Ile-Ser-Tyr-Gly-Arg-acetylated Lys-Lys-Arg-Arg-Gln-Arg-Arg-Arg-Ala-Pro (SEQ ID NO:19);

[0062] Gly-Leu-Gly-Ile-Ser-Tyr-Gly-Arg-acetylated Lys-Lys-Arg-Arg-Gln-Arg-Arg-Arg-Ser-Pro (SEQ ID NO:20);

[0063] Gly-Leu-Gly-Ile-Ser-Tyr-Gly-Arg-acetylated Lys-Lys-Arg-Arg-Gln-Arg-Arg-Arg-Pro-Pro (SEQ ID NO:21);

[0064] Gly-Leu-Gly-Ile-Ser-Tyr-Gly-Arg-acetylated Lys-Lys-Arg-Arg-Gln-Arg-Arg-Arg-Thr-His (SEQ ID NO:22);

[0065] Lys-Gly-Leu-Gly-Ile-Ser-Tyr-Gly-Arg-acetylated Lys-Lys-Arg-Arg-His-Arg-Arg-Arg-Thr-Pro-Gln (SEQ ID NO:23).

Atty. Dkt: UCAL296
Client Ref.: 2003-171-2

CLAIMS

What is claimed is:

1. An isolated acetylated immunodeficiency virus Tat polypeptide, wherein said polypeptide comprises at least one acetylated lysine residue.

2. The polypeptide of claim 1, wherein said polypeptide is a human immunodeficiency virus-1 Tat polypeptide, and wherein said acetylated lysine is Lys-50.

3. The polypeptide of claim 1, wherein said polypeptide comprises the amino acid sequence as set forth in any one of SEQ ID NOs:1-23.

4. The polypeptide of claim 1, wherein said polypeptide comprises the amino acid sequence Ser-Tyr-Gly-Arg-acetylated Lys-Lys-Arg-Arg-Gln-Arg-Cys (SEQ ID NO:03).

5. The polypeptide of claim 1, wherein said polypeptide comprises the amino acid sequence Ser-His-Gly-Arg-acetylated Lys-Lys-Arg-Arg-Gln-Arg-Cys (SEQ ID NO:04).

6. The polypeptide of claim 1, wherein said polypeptide is linked to a carrier.

7. An immunogenic composition comprising an acetylated immunodeficiency virus Tat polypeptide, wherein said polypeptide comprises at least one acetylated lysine residue; and a pharmaceutically acceptable excipient.

8. The immunogenic composition of claim 7, wherein said polypeptide is a human immunodeficiency virus-1 Tat polypeptide, and wherein said acetylated lysine is Lys-50.

9. The immunogenic composition of claim 7, wherein said polypeptide comprises the amino acid sequence as set forth in any one of SEQ ID NOs:1-23.

L52 ANSWER 12 OF 13 MEDLINE on STN

Citing References

88328963. PubMed ID: 2970960. Cellular processing of pro-atrial natriuretic factor (pro-ANF): studies using an antiserum that selectively binds ANF-(99-126) after its cleavage from pro-ANF. Wildey G M; Fischman A J; Fallon J T; Matsueda G R; Zisfein J B; Preibisch G; Seipke G; Homcy C J; Graham R M. (Cellular and Molecular Research Laboratory, Massachusetts General Hospital, Boston 02114.) Endocrinology, **(1988 Oct)** Vol. 123, No. 4, pp. 2054-61. Journal code: 0375040. ISSN: 0013-7227. Pub. country: United States. Language: English.

AB Atrial natriuretic factor (ANF) is stored in atrial myocytes as a 15-17K prohormone, but circulates in plasma as a 3K, carboxy (C)-terminal fragment of the prohormone. The tissue location at which the cleavage of pro-ANF to its hormonal form occurs is unknown. In the present study, an immunological approach was taken to address this question. A polyclonal antiserum was generated which recognizes the hormonal form of ANF [ANF-(99-126)] only after its cleavage from the prohormone. This was accomplished by immunizing rabbits with a synthetic **peptide** corresponding to the seven amino (N)-terminal residues of ANF-(99-126) coupled to **carrier** protein via a **C-terminal cysteine**. This antiserum, anti-ANF-(99-105), demonstrated high affinity for ANF-(99-126) (IC50 = 170 pM), but displayed 100-fold less affinity for recombinant pro-ANF [ANF-(2-126)]. The N-terminal specificity of anti-ANF-(99-105) was evident by its failure to bind ANF-(103-126) at concentrations up to 100 nM. The specificity of anti-ANF-(99-105) for the hormonal form of ANF was examined by using thrombin to cleave pro-ANF and testing for the generation of anti-ANF-(99-105) immunoreactivity. Cleavage of atrial pro-ANF or 35S biosynthetically-labeled pro-ANF resulted in the production of immunoreactive material from the prohormone, whereas pro-ANF itself demonstrated no cross-reactivity with anti-ANF-(99-105). Anti-ANF-(99-105) could also recognize ANF released from the isolated perfused rat heart. When anti-ANF-(99-105) was used in immunohistochemical studies of rat atrial myocardium, no staining was observed in unfixed frozen sections. This suggests that proteolytic processing of pro-ANF is not an intracardiocytic event.

PEP-CYS-CAR

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OTT, MELANIE
VERDIN, ERIC
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Lys Gly Leu Gly Ile Ser Tyr Gly Arg Xaa Lys Arg Arg His Arg Arg
1 5 10 15

Arg Thr Pro Gln
20

desirable to express the gene in eukaryotic cells, where the expressed protein will benefit from native folding and post-translational modifications. Small peptides can also be synthesized in the laboratory. Polypeptides that are subsets of the complete protein sequence may be used to identify and investigate parts of the protein important for function. Specific expression systems of interest include bacterial, yeast, insect cell and mammalian cell derived expression systems, which expression systems are well known in the art.

Acetylation

[0087] In some embodiments, a subject Tat polypeptide is acetylated *in vitro*, either after synthesis or during synthesis, or, e.g., after isolation from a naturally-occurring source of a Tat polypeptide. For example, where a subject Tat polypeptide is prepared synthetically *in vitro*, a Tat polypeptide is acetylated in a solution comprising 50 mM HEPES, pH 8, 10% glycerol, 1 mM DTT, 10 mM sodium butyrate, and 20 nmol acetyl-coenzyme A (AcCoA) in the presence of an acetyl transferase for 2 hours at 30°C. See, e.g., Ott et al. (1999) *Curr. Biol.* 9:1489-1492. An acetylated Tat protein can be generated as described in, e.g., Dorr et al. (2002) *EMBO J* 21:2715-2723; or Peloponese (1999) *J. Biol. Chem.* 274:11473-11478.

[0088] In other embodiments, a Tat polypeptide is acetylated by a living cell, e.g., the acetylated lysine is incorporated during synthesis of the Tat polypeptide. Tat acetylation in a eukaryotic cell is mediated by intracellular acetyltransferases, e.g., histone acetyl transferase (HAT), which catalyzes the transfer of an acetyl group from AcCoA to the epsilon amino group of lysine. Exemplary HATs include GCN5, MYST, p300/CBP, and nuclear receptors. Acetylated Tat polypeptide synthesized by a living eukaryotic cell is recovered using standard methods for protein purification. In some embodiments, the Tat polypeptide that is acetylated by a living eukaryotic cell is a fusion protein comprising a moiety that facilitates purification (e.g., a binding moiety), e.g., GST, 6His, etc., and the acetylated Tat polypeptide is purified using a separation medium appropriate to the binding moiety.

Compositions

[0089] The present invention provides compositions comprising a subject acetylated Tat polypeptide, which in some embodiments are immunogenic compositions. Compositions comprising a subject acetylated Tat polypeptide may include a buffer,

46-54

FIG. 5A

Tat alignment

46 AA

		1	10	20	30	40	50
	(1)	1	10	20	30	40	50
AAL29460	(1)	MEPVDPRLEPWKHPGSQPKTACTNCYCKKCCFHCQVCFITKALGISYGRK					
P04606	(1)	MEPVDPRLEPWKHPGSQPKTACTNCYCKKCCFHCQVCFITKALGISYGRK					
P04326	(1)	MEPVDPRLEPWKHPGSQPKTACTNCYCKKCCFHCQVCFITKALGISYGRK					
P04607	(1)	MEPVDPRLEPWKHPGSQPKTACTNCYCKKCCFHCQVCFITKALGISYGRK					
P04610	(1)	MEPVDPRLEPWKHPGSQPKTACTTCYCKKCCFHCQVCFTTKALGISYGRK					
P05908	(1)	MEPVDPRLEPWKHPGSQPKTACNNCYCKKCCYHCQVCFLTKGLGISYGRK					
P04608	(1)	MEPVDPRLEPWKHPGSQPKTACTNCYCKKCCFHCQVCFITKALGISYGRK					
P05907	(1)	MEPVDPRLEPWKHPGSQPKTACTNCYCKKCCFHCQVCFTKKALGISYGRK					
P19552	(1)	MEPVDPNLEPWKHPGSQPRTACTNCYCKKCCFHCQVCFITKGLGISYGRK					
P04609	(1)	MDPVDPNLEPWNHPGSQPKTACNRCHCKKCCYHCQVCFITKGLGISYGRK					
P12506	(1)	MDPVDPNIEPWNHPGSQPKTACNRCHCKKCCYHCQVCFITKGLGISYGRK					
P04611	(1)	MDPVDPNLEPWNHPGSQPRTPCNKCHCKKCCYHCPVCFLNKGLGISYGRK					
P18804	(1)	MDPVDPNLESWNHPGSQPRTACNKCHCKKCCYHCQVCFITKGLGISYGRK					
P04613	(1)	MDPVDPNLEPWNHPGSQPRTPCNKCYCKKCCYHCQMCFITKGLGISYGRK					
P24738	(1)	MEPVDPNLEPWKHPGSQPTTACSNCYCKVCCWHCQLCFLKKGLGISYGKK					
P04614	(1)	MEPVDPNLEPWKHPGSQPRTACNNCYCKKCCFHCYACFTRKGLGISYGRK					
P35965	(1)	MEPVDPNLEPWKHPGSQPRTACNNCYCKKCCFHCQVCFTKKGLGISYGRK					
P20893	(1)	MEPVDPRLEPWKHPGSQPKTASNNCYCKRCCLHCQVCFTKKGLGISYGRK					
P04612	(1)	MEPVDPRLEPWKHPGSQPKTACTTCYCKKCCFHCQVCFITKALGISYGRK					
P05905	(1)	MEPVDPRLEPWKHPGSQPKTACTTCYCKKCCFHCQVCFTKKALGISYGRK					
P05906	(1)	MDPVDPRLEPWKHPGSQPKAACTSCYCKKCCFHCQVCFTTKGLGISYGRK					
P19553	(1)	MEPVDPRLEPWKHPGSQPKTACTNCYCKKCCFHCQVCFITKGLGISYGRK					
P20879	(1)	MEPVDPSLEPWKHPGSQPKTACTNCYCKKCCLHCQVCFTTKGLGISYGRK					
Consensus	(1)	MEPVDPRLEPWKHPGSQPKTACTNCYCKKCCFHCQVCFITKALGISYGRK					

ACETYLATED TAT POLYPEPTIDES AND METHODS OF USE THEREOF
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54

FIG. 5B

KRRQRRRPPQGSQTHQVSLSKQPTSQSRG-DPT GPKESKKKVERETETDPFD- (SEQ ID NO: 42)
KRRQRRRPPQGSQTHQVSLSKQPTSQSRG-DPT GPKE---------- (SEQ ID NO: 43)
KRRQRRRAPQGSQTHQVSLSKQPTSQSRG-DPT GPKE---------- (SEQ ID NO: 44)
KRRQRRRPPQGSQTHQVSLSKQPTSQPRG-DPT GPKE---------- (SEQ ID NO: 45)
KRRQRRRPPQGSQTHQVSLSKQPTSQPRG-DPT GPKE---------- (SEQ ID NO: 46)
KRRQRRGPPQGSQTHQVSLSKQPTSQPRG-DPT GPKESKEKVERETETDPAVQ (SEQ ID NO: 47)
KRRQRRRAHQNSQTHQASLSKQPTSQPRG-DPT GPKE---------- (SEQ ID NO: 48)
KRRQRRRAHQDSQNHQASLSKQPSSQTRG-DPT GPKEPKKEVEREAETDPLD- (SEQ ID NO: 49)
KRRQRQRAPDSSQNHQDSLSKQPSSQPRG-DPT GPKESKKEVERETETDPLD- (SEQ ID NO: 50)
KRRQRRRPSQGGQTHQDPIPKQPSSQPRG-NPT GPKE---------- (SEQ ID NO: 51)
KRRQRRRPSQGGQTHQDPIPKQPSSQPRG-DPT GPKE---------- (SEQ ID NO: 52)
KRRQRRGPPQGGQAHQVPIPKQPSSQPRG-DPT GPKEQKKKVESEAETDP--- (SEQ ID NO: 53)
KRRQRRKPPQGDQAHQVPIPEQPSSQSRG-DPT GPKK---------- (SEQ ID NO: 54)
KRRQRRRPPQGNQAHQDPLPEQPSSQHRGDHPT GPKE---------- (SEQ ID NO: 55)
KRKPRRGPPQGSKDHQTLIPKQPLPQSQR-VSA GQEESKKKVESKAKTDRFA- (SEQ ID NO: 56)
KRRQRRRAPQDSQTHQASLSKQPASQSRG-DPT GPTESKKKVERETETDPFD- (SEQ ID NO: 57)
KRRQRRRPPQDSQTHQSSLSKQPTSQLRG-DPT GPTESKKKVERETETDPVH- (SEQ ID NO: 58)
KRRQRRRAPQDSKTHQVSLSKQPASQPRG-DPT GPKESKKKVERETETDPED- (SEQ ID NO: 59)
KRRQRRRA---------- ---------- (SEQ ID NO: 60)
KRRQRRRAPEDSQTHQVSLPKQPAPQFRG-DPT GPKESKKKVERETETHPVD- (SEQ ID NO: 61)
KRRQRRRAPQDSQTHQVSLPKQPASQARG-DPT GPKESKKKVERETETDPVD- (SEQ ID NO: 62)
KRRQRRRAPPDSEVHQVSLPKQPASQPQG-DPT GPKESKKKVERETETDPVH- (SEQ ID NO: 63)
KRRQRRRPPQDSQTHQVSLPKQPSSQQRG-DPT GPKESKKKVERETETDPDN- (SEQ ID NO: 64)
KRRQRRRPPQGSQTHQVSLSKQPTSQSRG-DPT GPKESKKKVERETETDPFD- (SEQ ID NO: 65)

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Acetylation of HIV-1 Tat by CBP/P300 Increases Transcription of Integrated HIV-1 Genome and Enhances Binding to Core Histones

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The HIV-1 Tat protein is required for viral replication and is a potent stimulator of viral transcription. Although Tat has been extensively studied in various reductive paradigms, to date there is little information as to how this activator mediates transcription from natural nucleosomally packaged long terminal repeats. Here we show that CREB-binding protein (CBP)/p300 interacts with the HIV-1 Tat protein and serves as a coactivator of Tat-dependent HIV-1 gene expression on an integrated HIV-1 provirus. The site of acetylation of Tat was mapped to the double-lysine motif in a highly conserved region, ^{49}RKKRRQ^{54}, of the basic RNA-binding motif of Tat. Using HLM1 cells (HIV-1$^+$/Tat$^-$), which contain a single copy of full-length HIV-1 provirus with a triple termination codon at the first AUG of the Tat gene, we find that only wild type, and not K50A, K51A, or K50A/K51A alone or in combination of ectopic CBP/p300, is able to produce full-length infectious virions, as measured by p24 gag ELISAs. Tat binds CBP/p300 in the minimal histone acetyltransferase domain (1253–1710) and the binding is stable up to 0.85 M salt wash conditions. Interestingly, wild-type peptide 41–54, and not other Tat peptides, changes the conformation of the CBP/p300 such that it can acquire and bind better to basal factors such as TBP and TFIIB, indicating that Tat may influence the transcription machinery by helping CBP/p300 to recruit new partners into the transcription machinery. Finally, using biotinylated wild-type or acetylated peptides, we find that acetylation decreases Tat's ability to bind the TAR RNA element, as well as to bind basal factors such as TBP, CBP, Core-Pol II, or cyclin T. However, the acetylated Tat peptide is able to bind to core histones on a nucleosome assembled HIV-1 proviral DNA. © 2000 Academic Press

INTRODUCTION

The HIV-1 Tat protein is required for viral replication and is a potent stimulator of viral transcription. Tat stimulates viral gene expression through an RNA element in the viral long terminal repeat (LTR). For optimal transactivation of HIV-1 gene expression, Tat requires specific upstream transcription factors, including Sp1 (Jeang *et al.*, 1993), TATA-binding protein (Kashanchi *et al.*, 1994b; Veschambre *et al.*, 1995; Majello *et al.*, 1998), Tat-associated kinase (Herrmann and Rice, 1995; Yang *et al.*, 1996), TFIIH (Garcia-Martinez, *et al.*, 1997; Parada and Roeder, 1996), Tip (Jeang *et al.*, 1993; Henderson *et al.*, 1999), and RNA polymerase II (Cujec *et al.*, 1997; Mavankal *et al.*, 1996). The ability of Tat to regulate viral transcription is related to its ability to interact with the basal transcription complexes responsible for the initiation of transcription including cyclinT_1/cdk9 complex, resulting in a more efficient elongating RNA Pol II complex (O'Keeffe *et al.*, 2000; Romano *et al.*, 1999; Napolitano *et al.*, 1999; Isel and Karn, 1999; Bieniasz *et al.*, 2000; Ramanathan *et al.*, 1999; Ivanov *et al.*, 1999; Chen *et al.*, 1999a; Wimmer *et al.*, 1999; Garriga *et al.*, 1998; Garber *et al.*, 1998; Fujinaga *et al.*, 1998; Wei *et al.*, 1998).

Among the factors associated with basal transcription complexes, CBP (CREB-binding protein) and p300 have emerged as coactivators for various DNA-binding transcription factors. CBP and p300 are large proteins, 2441 and 2414 amino acids, respectively, that have the ability to interact simultaneously with various transcription factors such as nuclear hormone receptors, CREB, c-Jun, v-Jun, c-Myb, v-Myb, Sap-1a, c-Fos, MyoD, YY1, NF-κB, and p53 (Goldman *et al.*, 1997) and with other coactivators such as P/CAF (Blanco *et al.*, 1998; Chakravarti *et al.*, 1999), as well as with basal components of the transcriptional apparatus. Therefore, it is this wide array of functions that have allowed CBP/p300 proteins to be important transcriptional integrators (Shikama *et al.*, 1999). In recent years multiple mechanisms have emerged for the function of CBP/p300. The first mechanism for CBP/p300 activation involves the acetylation of the terminal tails of the core histones by histone acetyltransferase (HAT) and destabilization of histone–DNA interactions, allowing transcription factors access to the promoter region. The second mechanism by which CBP has been suggested to be an activator of transcription is by bridging the gap between upstream DNA-bound transcription factors and

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components of the general transcription machinery. The third possible mechanism is CBP/p300's ability to directly acetylate nonhistone proteins such as p53 (Gu and Roeder, 1997), the erythroid Kruppel-like factor (EKLF, Zhang and Bieker, 1998), the nuclear hormone receptor coactivators ACTR (Chen *et al.*, 1997), and the basal transcription factor TFIIE and TFIIF (Imhof *et al.*, 1997; Martinez-Balbas *et al.*, 1998). In the case of p53, acetylation of the regulatory domain led to a dramatic increase in DNA binding *in vitro*, whereas the acetylation of ACTR by CBP/p300 disrupts the receptor–coactivator interaction, which plays a key role in hormone-induced gene activation (Chen *et al.*, 1999b).

HIV-1 proviral DNA is integrated into host cell chromosomes and packaged into chromatin. The LTR acts as a very strong promoter when analyzed as naked DNA *in vitro* and is silent when integrated into the cellular host genome in the absence of any stimuli (Verdin, 1991; Adams *et al.*, 1994; Van Lint *et al.*, 1996; Marzio *et al.*, 1998; Benkirane *et al.*, 1998). Recently, several reports have shown the existence of an intracellular multiprotein complex that contains Tat, CBP/p300, and P/CAF. It was found that the histone acetyltransferase activity of CBP/p300 and P/CAF is preferentially required for Tat function (Kiernan *et al.*, 1999). CBP/p300 was also recently reported to interact with the HIV-1 Tat protein and serves as a coactivator of Tat-dependent HIV-1 gene expression (Hottiger *et al.*, 1998; Ott *et al.*, 1999). This superinduction has been attributed to the histone acetyltransferase (HAT) activity of CBP/p300 on the integrated HIV-1 promoter.

In this study, we find that Tat is acetylated by CBP/p300 and mapped to the double-lysine motif in a highly conserved region (^{49}RKKRRQ^{54}) of the Tat protein. Using HLM1 (HIV-1$^+$/Tat$^-$) cells, which contain a single copy of full-length HIV-1 provirus with a triple termination codon at the first AUG of the Tat gene, we find that only wild type, and not K50A, K51A, or a double-mutant K50A/K51A alone or in combination with excess CBP/p300, is able to produce full-length infectious virions. Furthermore, mechanistically, the wild-type Tat peptide 41–54, which contains the basic core domain of HIV-1 Tat, changes the conformation of CBP/p300 such that basal factors such as TBP and TFIIB bind better to CBP/p300, indicating that Tat may influence the transcription machinery by helping CBP/p300 to acquire new partners in the transcription machinery.

Tat binds to CBP/p300 minimal HAT domain (1253–1710) and is a stable complex up to 0.85 M salt wash conditions. Acetylation of Tat by CBP/p300 decreases Tat's ability to bind the TAR RNA element *in vitro*. Finally, using biotinylated wild-type or acetylated Tat peptides, we find that acetylation causes a release of Tat from basal factors such as TBP, CBP, or cyclin T. Interestingly, the acetylated Tat peptide is able to bind with higher affinity to core histones on nucleosomal DNA.

RESULTS

HIV-1 Tat is acetylated by the HAT domain of CBP/p300

We initially asked whether a minimal HAT domain of CBP/p300 was capable of acetylating the Tat protein *in vitro*. To answer this question, we constructed a minimal GST–HAT plasmid from a full-length p300 cDNA clone, expressed in *Escherichia coli* and purified using glutathione–agarose beads. When using GST–HAT in a [^{14}C]acetyl coenzyme A exchange reaction, we found that GST–HAT was capable of efficient acetylation of core histones H2A, H2B, H3, and H4 *in vitro* (Fig. 1A, lane 3). We also observed efficient acetylation of purified Tat protein *in vitro*, as shown in Fig. 1A, lane 4. Products shown in Fig. 1A are ^{14}C-acetylated polypeptides that had been separated on 4–20% SDS–PAGE, dried, and exposed to a PhosphorImager cassette. The bottom panel of Fig. 1A shows the Coomassie blue staining of the same gel. It is important to note that we have consistently observed a more efficient acetylation of Tat proteins that do not contain a GST moiety at their N-terminus. Similar efficient *in vitro* acetylations were also observed with histidine- and epitope (influenza)-tagged peptides at the N- or C-terminus of the Tat protein (data not shown).

We next examined the effect of various Tat peptides as substrates in the *in vitro* HAT assay. The results of such an experiment are shown in Fig. 1C, where Tat 41–54 peptide, but not 41–50 peptide (lanes 7–10), was acetylated with GST–HAT. Similar to Fig. 1A, results shown in Fig. 1C are products separated on 4–20% SDS–PAGE, dried, and exposed to a cassette. It is important to note that we have observed reproducible results only when using [^{14}C]acetyl CoA and SDS–PAGE (4–20%) for separation purposes as opposed to [^{3}H]acetyl CoA and filter disks for detection of acetylated small peptides. Peptides of such short lengths do not reproducibly bind to DE52 filter papers and cannot stand rigorous wash conditions. A summary of all the Tat peptides used in the HAT assay is shown in Fig. 1D. It is important to note that peptides such as 65–86, which contains lysine residues, were not acetylated, indicating that the *in vitro* acetylation by the CBP/P300 HAT domain is not a nonspecific reaction. Finally, we used wild-type full-length human HeLa p300 (hp300, a generous gift from R. Shiekhattar), or epitope-tagged recombinant p300 (rp300), and observed efficient acetylation of the Tat protein *in vitro* (Fig. 1E).

Site of Tat acetylation by the HAT domain

The Tat peptide 41–54 contains three lysine residues, one at position 41 and the other two at positions 50 and 51. To determine which lysine residues in the 41–54 peptide were acetylated by GST–HAT, we initially made acetylated 41–54 peptides with acetyl groups at posi-

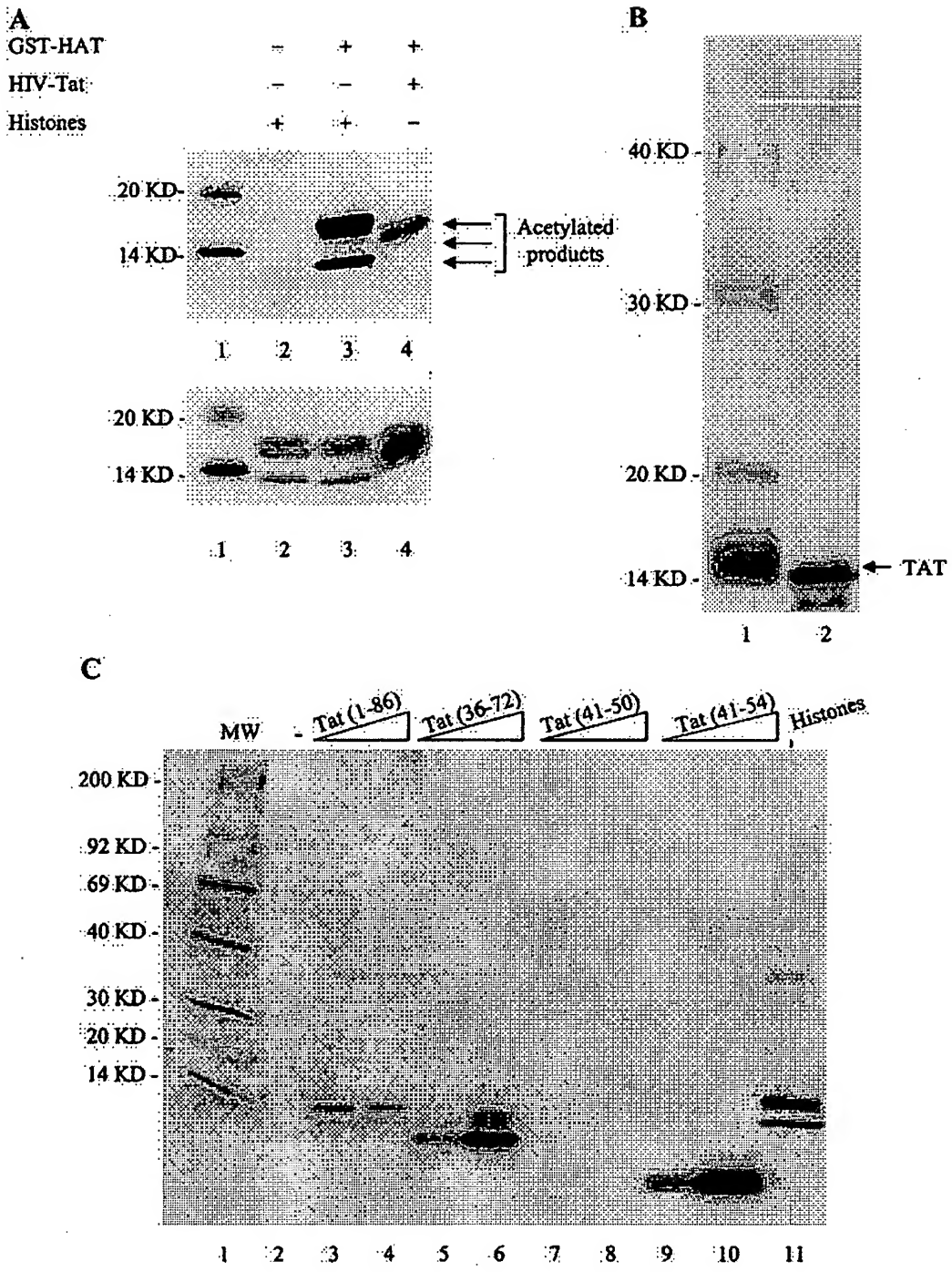


FIG. 1. Acetylation of HIV-1 Tat protein by CBP/p300 *in vitro*. (A, B) The core histones H2A, H2B, H3, and H4 (lanes 2 and 3) and Tat protein (1–86, lane 4) were incubated with or without GST–p300 (HAT domain) and [^{14}C]acetyl-CoA. (A) Acetylated products (lanes 3 and 4) resolved on 4–20% SDS–PAGE, dried, and exposed to a PhosphorImager cassette. (A, bottom) Coomassie blue staining of the gel shown at top. B represents the purified full-length Tat protein (1–86, 1 μg) used in the HAT assay, resolved on a SDS–PAGE gel, and silver stained (31). (C) Tat acetylation site located at the basic RNA-binding domain. Synthesized Tat peptides covering various regions of the Tat protein (lane 3–11) were incubated with GST-p300 and [^{14}C]acetyl CoA and analyzed on 4–20% SDS–PAGE. Lane 2 serves as negative control with no substrate added to the reaction, and lanes 3, 4, and 11 serve as positive controls, where full-length Tat and core histone proteins were added to the reaction. All other reactions were performed with two concentrations (200 and 400 ng) of various peptides. (D) The schematic representation of Tat protein and Tat peptides used in this study and the results of acetylation by GST–p300. (E) Acetylation of HIV-1 TAT by wild-type p300/CBP. (Left) Acetylation of TAT or all four histones with 10 ng of HeLa purified CBP/p300 complexes (hp300). Proteins, BSA, wild-type TAT, or histones were incubated with HeLa CBP/p300, followed by incubation at 37°C for 1 h, spotted on DE52 filter discs, washed, and counted. (Right) A similar experiment with either TAT or free histones incubated with 10 ng of purified epitope-tagged recombinant Baculovirus p300 (rp300). Samples were incubated at 37°C for 1 h and subsequently run on 4–20% SDS–PAGE, dried, and exposed to a PhosphorImager cassette.

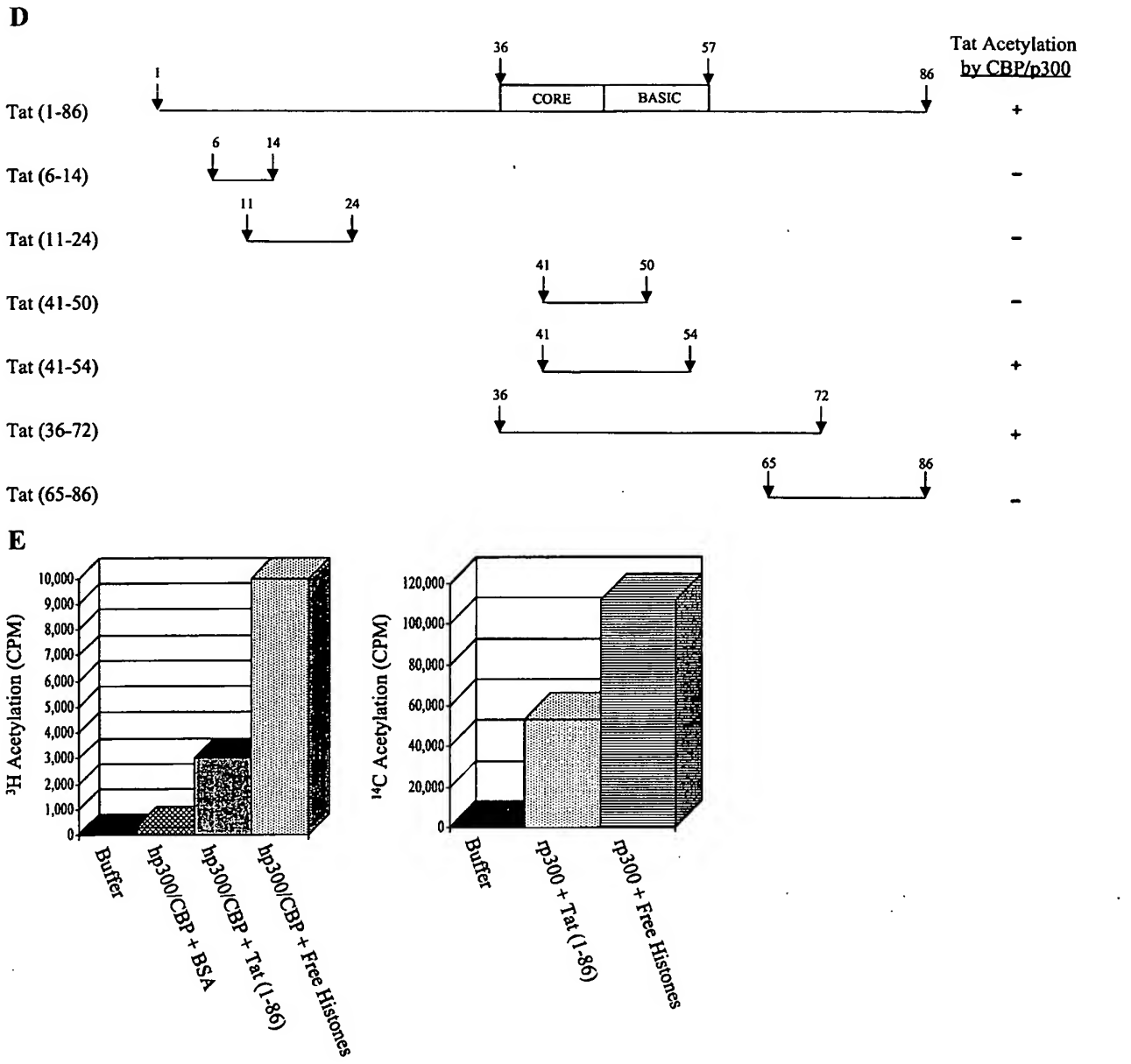


FIG. 1—*Continued*

tions 50, 51, or 50+51. The acetyl groups were put on the peptides during the chemical peptide synthesis. All peptides were subsequently purified on C18 reverse-phase HPLC and dried, and quantitations were determined by protein assay as well as by running small aliquots on 4–20% SDS–PAGE followed by silver stain detection. As can be seen in Fig. 2A, all peptides were efficiently acetylated with GST–HAT, except the double-acetylated 41–54 (lanes 9 and 10). The doubly acetylated 41–54 peptide was, however, very weakly acetylated at lysine 41 position, at a 10-fold excess of GST–HAT concentrations *in vitro,* and similar results were also obtained with a longer peptide, 36–58 (data not shown). Therefore, it may be possible that all three lysines are acetylated, although at very different kinetic rates, depending on the enzyme concentrations used in these assays. To determine which lysine, 50 or 51, was acetylated efficiently *in vitro,* we synthesized a second batch of acetylated peptides starting from position 42–54. The results of such an experiment are shown in Fig. 2B, where peptide 42–54, which had already been acetylated at position 50, was a poor substrate for accepting the new acetyl group at position 51 (lanes 3 and 4). However, the peptide that was acetylated at position 51 could serve as an excellent substrate for acetylation *in vitro.* The doubly acetylated 42–54 peptide could not be acetylated at any concentration of GST–HAT (data not shown). We next examined the effect of Tat acetylation on cells expressing Tat. HeLa/eTat and HeLa/pcep4 lines contain either an epitope-tagged Tat at the C-terminus (HeLa/eTat) or a control

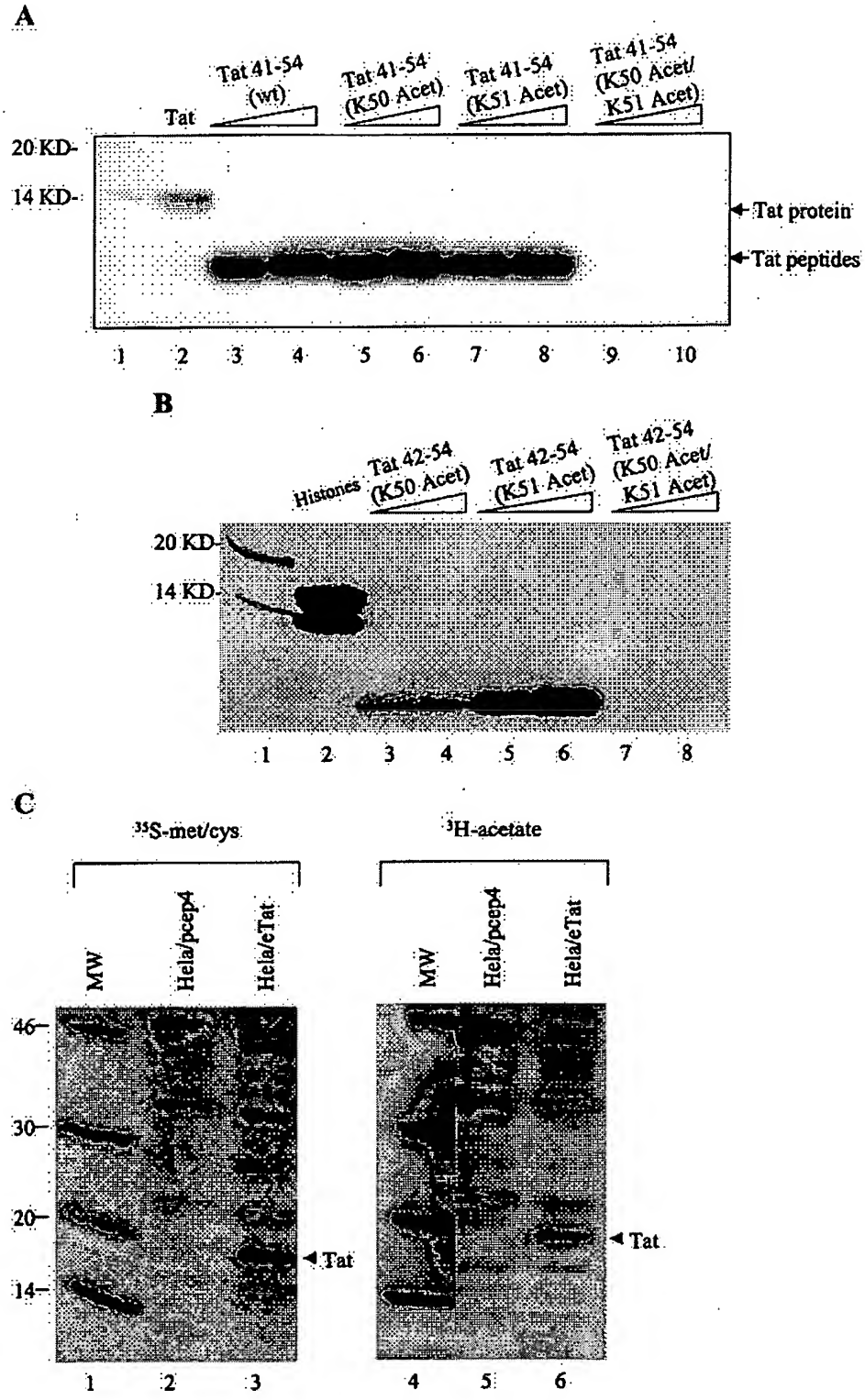


FIG. 2. Tat acetylation sites mapped to double-lysine motifs K50 and K51 in the basic RNA-binding domain. (A, B) Two concentrations (200 and 400 ng) of the Tat peptides (41–54 or 42–54), wild-type or acetylated at lysines position 50, 51, and 50+51, were incubated with GST–p300 and [^{14}C]acetyl CoA, separated on 4–20% SDS–PAGE, and exposed to a PhosphorImager cassette. (C) Both log-phase HeLa/eTat or HeLa/pcep4 cells were labeled with [^{3}H]acetate in DMEM complete medium plus hygromycin (left) or incubated overnight with [^{35}S]methionine/cysteine (right). Nuclear lysates were used for immunoprecipitations on cross-linked monoclonal 12CA5 Ab beads and eluted with a 100-fold excess of influenza peptide. The [^{35}S]methionine/cysteine gel was exposed overnight and the [^{3}H]acetate gel was exposed for 1 week on the PhosphorImager cassette.

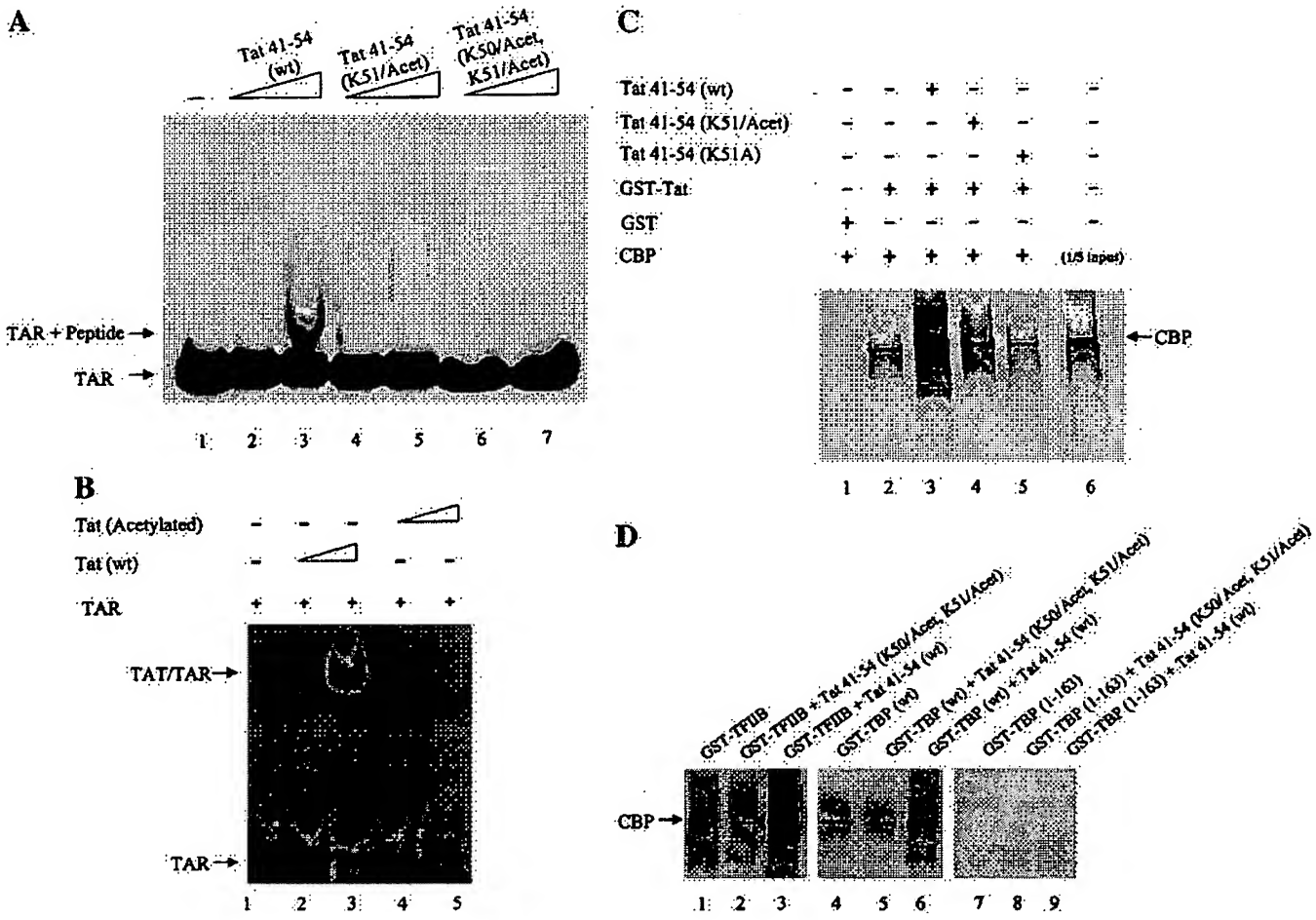


FIG. 3. Acetylated Tat decreases its ability to HIV-1 TAR RNA. (A) The wild-type Tat peptide 41–54 (lanes 2 and 3) and the acetylated Tat peptide 41–54 (lanes 4–7) were incubated with ^{32}P-labeled TAR RNA at room temperature for 30 min and separated on a 6% DNA retardation polyacrylmide gel (Novex). Lane 1 contains TAR RNA alone and lanes 2–7 indicate TAR plus various wild-type or acetylated peptides (2.5 and 5 μg). (B) Wild-type (lanes 2 and 3, 0.5 and 1.0 μg, respectively) or GST–HAT *in vitro* acetylated Tat proteins (1–86, lanes 4 and 5, 0.5 and 1.0 μg, respectively) were incubated with TAR RNA, resolved an a 6% DNA retardation gel, dried, and exposed to a PhosphorImager cassette. (C, D) Tat peptide (41–54) increases the binding of CBP to TBP, TFIIB, and Tat. Tat peptides, wild type or various modified versions (10 μg), were preincubated with ^{35}S-labeled CBP (Promega TNT, 10 μl) for 10 min and subsequently used to bind to 500 ng eluted GST–Tat, GST–TFIIB, GST–TBP (wt), or GST–TBP (1–163, deletion from 164 to 337 of human TBP). After several washes (3×), the bound proteins were resolved by 4–20% SDS–PAGE, dried, and exposed.

backbone vector (HeLa/pcep4). Both cell types have been described previously (Kashanchi *et al.*, 2000). Log-phase-growing cells were labeled with [^{3}H]acetate or [^{35}S]methionine/cysteine, and nuclear extracts were obtained for immunoprecipitations on cross-linked 12CA5 antibody beads. Following binding, Tat was eluted with an excess of competitor peptide and run on a 4–20% gel. The results of such an experiment are shown in Fig. 2C, where acetylated Tat (right column) could be obtained only from HeLa/eTat cells and not from the control pcep4 cells.

Effect of Tat acetylation on TAR and CBP/P300 binding

We next asked whether acetylation of Tat could increase or decrease its affinity for TAR RNA. To address this question, we synthesized labeled TAR RNA, PAGE purified it, and used it in an RNA bandshift assay. The results of such an experiment are shown in Fig. 3A, where wild-type peptide 41–54 was capable of binding to TAR RNA (lane 3). The TAR RNA binding is completely abolished when lysines 50 and 51 are acetylated (lanes 6 and 7). We observed no binding of double-acetylated 50 and 51 peptide to TAR RNA at any peptide concentration (data not shown). Similar results were also observed when Tat protein was acetylated with GST–HAT prior to TAR RNA binding (Fig. 3B, lanes 4 and 5).

We next examined the effect of wild-type or acetylated peptides on CBP/p300 binding *in vitro*. We reasoned that if only wild-type Tat peptide or protein was able to bind to TAR RNA and not the acetylated counterparts, it would then be possible for acetylated Tat to also bind less efficiently to CBP/p300. The results of such an experiment are shown in Fig. 3C, where GST–Tat, but not GST,

was able to bind to ^{35}S-labeled CBP *in vitro*. However, when performing competition experiments with wild-type, acetylated, or alanine-substituted Tat peptides in the same reaction, we found a surprising result, where wild-type 41–54 but not other derivatives was able to enhance the binding of CBP to GST–Tat (compare lanes 3–5). We have found similar results when using immunoaffinity-purified recombinant p300 from Baculovirus-infected cells (data not shown). This unexpected result suggested to us that perhaps the wild-type peptide might change the conformation of CBP/p300 such that it can bind better to other proteins. This interpretation is very likely, since Tat has been shown to dimerize and contact multiple transcription factors on the transcription initiation site. To test that hypothesis, we performed a similar experiment with CBP pretreated with either wild-type or double-acetylated peptide prior to binding to other basal factors such as TFIIB and TBP. Both TFIIB and TBP have previously been shown to bind to CBP/p300 molecules (Sang *et al.*, 1997). The results in Fig. 3D indicate that when CBP is pretreated with only wild-type Tat peptide, it can bind more efficiently to GST–TFIIB or GST–TBP, but not to a GST–TBP (1–163) mutant. The reaction was specific for a possible change of CBP conformation, since pretreatment of GST–TFIIB, TBP, or the mutant TBP with any of the peptides did not increase CBP binding *in vitro* (data not shown). Taken together, the results from TAR binding as well as CBP binding indicate that wild-type and not acetylated Tat binds to the basal transcription machinery and that acetylated Tat might either completely come off the transcriptional complex or simply stay with the elongating RNA Pol II.

Localization of CBP/p300 binding to Tat

It has previously been shown that p300 binds to Tat at core and basic residues (Marzio *et al.*, 1998; Benkirane *et al.*, 1998; Hottiger and Nabel, 1998) and that Tat binds to the HAT, N-terminal, or C-terminal domains of p300. We decided to determine which region of p300 was stably binding to the Tat protein. Initially, we used a series of four GST–CBP mutants (a generous gift from R. Goodman) and three GST–p300 mutants in an *in vitro* TNT-binding assay. Figure 4A is a general diagram that depicts the relationship between CBP and p300 molecules as well as various mutants used in our assays. When binding ^{35}S-labeled Tat to various GST–p300 domains, we found that Tat bound stably to all A, B, and C mutant domains under 0.1 M salt wash conditions (Fig. 4B, lanes 1–4). However, the binding of Tat to p300 was most resistant with the B fragment (aa 744–1540) of GST–p300. Tat retained its binding to this fragment under 0.85 M salt wash conditions (compare lanes 12–14). Similar results were obtained with the GST–CBP HAT domain (data not shown). Therefore, Tat may interact with multiple CBP/p300 domains; however, its ability to tightly associate with the HAT domain may indicate similarity in functions with other viral HAT-binding proteins such as adenovirus E1A or SV40 large T antigen.

Functional activity of mutant Tat proteins on transient transfections

Tat is one of the most powerful viral activators known to date. Tat could stimulate transcription of HIV-1 promoter anywhere from 100- to 1000-fold depending on the assay conditions used. To determine which Tat residues (position 50, 51, or 50+51) were important for HIV-1 promoter activity, we synthesized alanine-substituted vectors and sequenced all three forms of CMV-driven Tat vectors. We subsequently used the Tat mutants in transient transfection assays using CEM (12D7) T-cells. The results of such an experiment are shown in Fig. 5A, where upon electroporation of K50A or K51A with reporter HIV-LTR CAT, we observed a slight drop in transcriptional activity (less than 50%, compare lanes 3–6). However, a more pronounced drop in transactivation was observed with the double-mutant 50 and 51 (~5-fold, lanes 7 and 8). This drop in activation from the 50+51 mutant could not be rescued with ectopic expression of CBP vector (Fig. 5B, lane 4). Similar results were obtained with a CMV–p300 expression vector (data not shown). Finally to conserve the positive charge of the lysine residues, we synthesized Tat protein with arginines at positions 50 and 51 and used the protein to perform CAT assays in CEM cells. The results in Fig. 5C indicate that only lysine residues and not alanine or arginine substitutions at positions 50 and 51 are critical for the observed acetylation effect. Taken together, the transient transfection results indicate that neither K50 nor K51 mutations alone are sufficient to completely lose the Tat transactivation on HIV-1 LTR.

Activation of integrated HIV-1 provirus requires the HAT domain of CBP/P300

We used HLM-1 cells to address whether the interaction of Tat and CBP/p300 plays a role in the activation of integrated proviral HIV-1 sequence *in vivo*. HLM-1 cells (AIDS Research and Reference Reagent Program) were derived from HeLa-T4$^+$ cells containing an integrated copy of the HIV-1 proviral genome with the Tat-defective mutation (termination linker at the first AUG). HLM-1 cells are negative for virus particle production, but can be induced to express high levels of infectious HIV-1 after transfection with Tat-expressing clones or after stimulation with cytokines such as TNF-α or general inducers such as sodium butyrate. The new resulting particles are wild type for infection, RT activity, and integration, but need to be restimulated for the next round of progeny formation. In order to test whether coactivator CBP/p300 plays an important role in the activation of integrated HIV-1, we transfected the HLM-1 cells with Tat and CBP

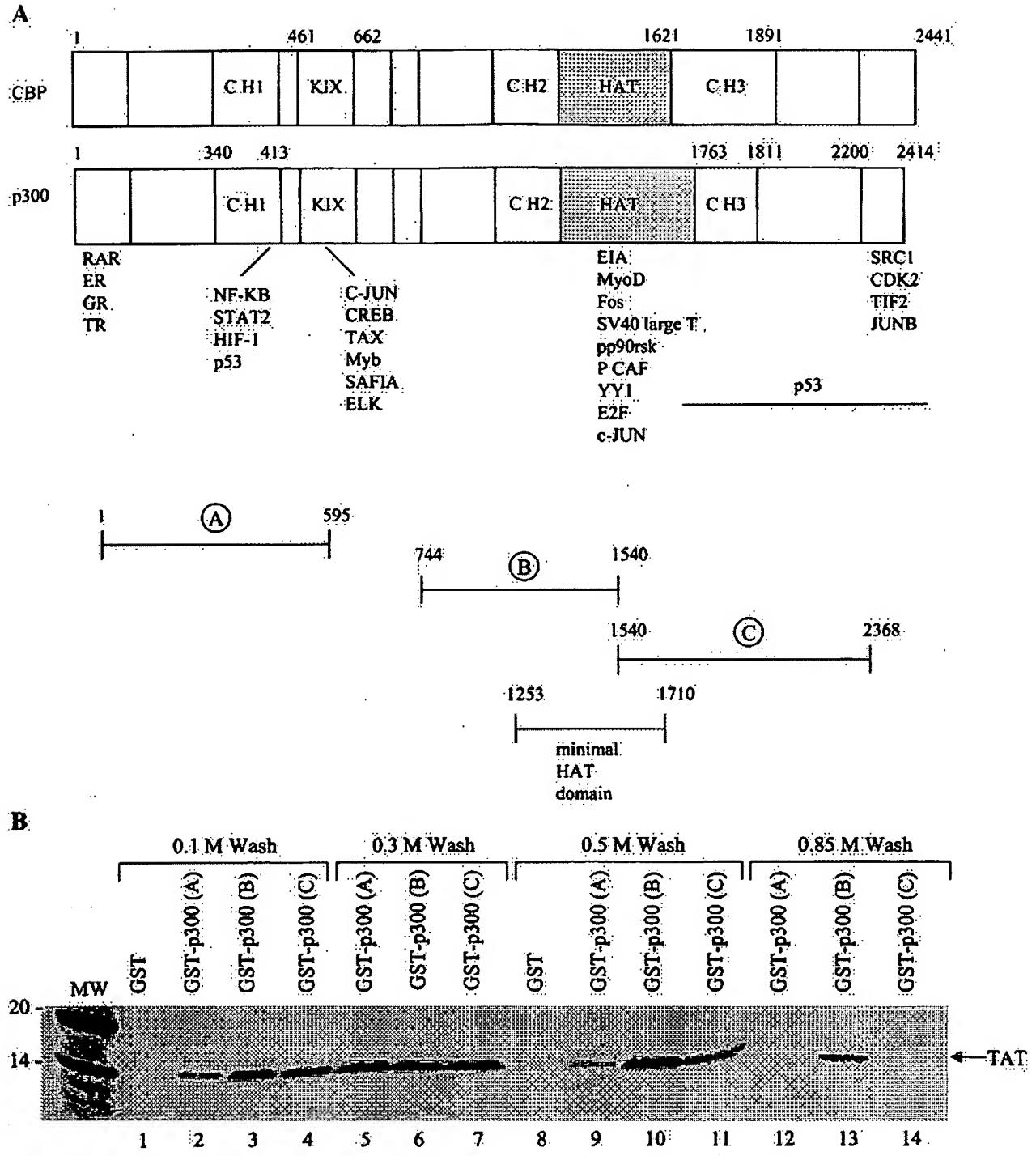
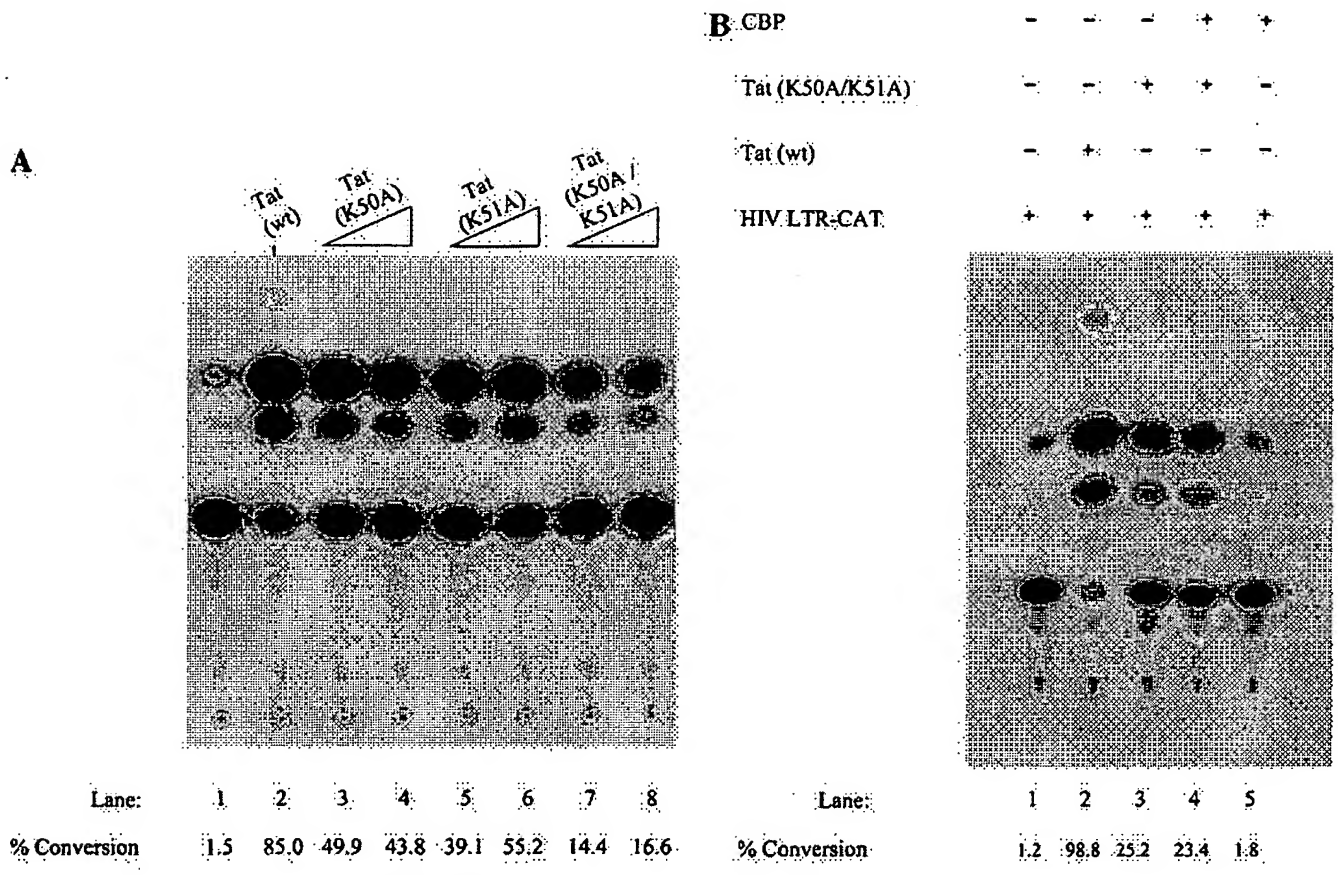


FIG. 4. Binding of Tat to CBP and p300 under various salt conditions. (A) Schematic representation of functional domains in CBP, p300, and the GST–p300 clones containing various domains used here, including GST–p300 A (1–595), GST–p300 B (744–1540), and GST–p300 C (1540–2368). (B) Binding of Tat to p300 under various salt wash conditions. The GST–p300 deletion clones (A, B, and C) were immobilized on glutathione beads from bacterial extracts and incubated with radiolabeled, *in vitro* translated ^{35}S-labeled Tat. The bound proteins were resolved on 4–20% SDS–PAGE after being washed with buffers containing TNE + 0.1, 0.3, 0.5, or 0.85 M salt and 0.1% NP-40. Tat binds to GST–p300 B and C fragments at 0.3 M salt buffer. Tat binds only to GST–p300 B fragment after a 0.85 M salt wash buffer.

and with or without E1A, which has been shown to inhibit the HAT activity of CBP/p300 (Chakravarti *et al.*, 1999). The resulting supernatants were collected at various time points and tested for the production of HIV-1 particles by p24 gag antigen ELISA. The results of such an experiment are shown in Fig. 6A, where ectopic expression of CBP along with Tat activates the viral production by four- to fivefold. Furthermore, the effect of CBP acti-


A
Tat (wt)
Tat (K50A)
Tat (K51A)
Tat (K50A / K51A)
Lane: 1 2 3 4 5 6 7 8
% Conversion 1.5 85.0 49.9 43.8 39.1 55.2 14.4 16.6
B
CBP - - - + +
Tat (K50A/K51A) - - + + -
Tat (wt) - + - - -
HIV LTR-CAT + + + + +
Lane: 1 2 3 4 5
% Conversion 1.2 98.8 25.2 23.4 1.8
C
TAT (wt) - - - +
TAT (K50/51R) - - + -
TAT (K50/51A) - + - -
HIV LTR-CAT + + + +
1 2 3 4
% Conversion 0.9% 17% 9.2% 88%

vation can be reversed using the E1A expression vector. Interestingly, neither E1A nor CBP by itself can activate HIV-1 production, indicating at least in the case of CBP, that CBP exerts its activation effect on the HIV-1 promoter only in the context of chromatin DNA. Therefore, unlike other retroviral activation pathways, such as HTLV-1 (Kashanchi *et al.*, 1998), the HAT domain of CBP/p300 may be a crucial domain in HIV-1 proviral transcriptional activation.

We next examined the effect of the Tat mutants at positions 50 and 51 and a combination 50+51 mutant in HLM1 cells. Results presented in Fig. 6B indicate that point mutations at position 50 or 51 are equally deleterious in activation of the integrated chromatinized HIV-1 DNA. This is in marked contrast to the transfection data (Fig. 5A) where point mutants at 50 or 51 were slightly affected and only the double-mutant 50+51 vector was dramatically reduced in activity. More importantly, ectopic expression of the CBP could not activate the 50 or 51 mutants in these cells. Similar levels of nuclear Tat wild type and mutants were observed in these cells (data not shown). Finally, we performed a titration assay of the Tat 50+51 mutant with CBP and found no induction of the latent virus in these cells (Fig. 6C). Similar results were also obtained with the Tat point mutants 50 or 51 and titration of various concentrations of ectopically expressed CBP (data not shown). Taken together these data indicate that the effect of CBP/p300 is at the level of integrated HIV-1 provirus and that both lysines at position 50 and 51 are equally important for this activation pathway.

Binding target of Tat acetylated peptide

To date there are no clear examples of what exactly happens to a protein once it is acetylated in the transcriptional complex by coactivators such as CBP/p300. In fact, the majority of the existing reports on acetylated proteins discuss what the functional consequence of acetylation may be (Gu and Roeder, 1997) and not so much regarding the proteins or pathways downstream of acetylation. We decided to address this question by utilizing affinity pull-down experiments using wild-type or acetylated 42–54 peptides coupled to a biotin moiety. The biotinylated peptides were incubated with various ^{35}S-labeled proteins or nuclear extracts followed by direct radioactive detection or Western blot for transcription factors involved in HIV-1 transcription. The results of such an experiment are shown in Fig. 7A, where ^{35}S-labeled TBP, CBP, and cyclin T could efficiently bind to wild-type but not acetylated 42–50 peptide. A similar experiment was performed using a purified HeLa core-Pol II fraction (Inostroza *et al.*, 1994; Piras *et al.*, 1994), followed by Western blot for Pol II. Results from Western blots indicated that wild-type and not acetylated peptide was capable of binding to core-Pol II. In an attempt to find substrates that could bind to acetylated peptide, we performed a series of binding assays followed by excision of bound peptides from gels and subjected them to mass spectrometry. The initial material for pull-down assays was whole HIV-1 virus (pDH125, a generous gift from M. Chow and M. Martin) reconstituted *in vitro* with all four histones. The bound complexes were washed with 150 mM salt and 0.5% NP-40, run on SDS–PAGE, and silver-stained, and peptides were excised and subjected to mass spectrometry. The results of such an experiment are shown in Fig. 7B, where acetylated Tat peptide was able to bind to core histones. It is interesting to note that core histones in the absence of DNA did not bind to acetylated Tat, indicating that a fixed conformation of nucleosome is required for Tat to bind to core histones. Therefore, these data collectively suggest that acetylated Tat may leave the initiation complex behind and retain its binding to nucleosomal DNA.

DISCUSSION

In recent years it has become apparent that non-DNA-binding transcriptional coactivators, such as p300 and CBP, that were previously thought to function primarily as "bridging" proteins between DNA-bound transcription factors and the basal transcription complex play a critical regulatory role as integrators of diverse signaling pathways in the selective induction of gene expression. Many examples of such phenomena are exemplified by CBP/p300's interaction with an array of transcription factors including sequence-specific DNA-binding proteins, such as the NF-κB CREB, or activator protein 1 (AP-1) family members, that interact with the promoter and act as

FIG. 5. Mutation of the lysine residues at position 50 and 51 of Tat and their reduced LTR transactivation activity. (A) CAT assays were performed from lysates of transfected CEM (12D7) cells with LTR-CAT (3 μg) and varying concentrations of CMV-Tat mutants (1 and 5 μg). Lanes 1 and 2 represent basal transcription of LTR-CAT and Tat (1 μg) activated transcription, respectively. Lanes 3–8 show transfection of single- or double-mutant Tat constructs into CEM cells. The mutants were pcTat (K50A, lanes 3, and 4), pcTat (K51A, lanes 5 and 6), and pcTat (K50A/K51A, lanes 7 and 8). Cells were transfected by the electroporation method and processed for CAT assay 24 h later. The percentage of CAT conversion is indicated at the bottom of each lane. (B) Cotransfection of pcTat (K50A/K51A) alone or with CBP (2.5 μg) in CEM cells is shown in lanes 4 and 5, respectively. Lanes 1 and 5 serve as negative controls. Titration of either pcTat (K50A/K51A) or CBP plasmids showed similar results, where CBP was not able to rescue the Tat mutant construct (data not shown). (C) Transfection of various proteins along with LTR-CAT reporter into CEM cells. CEM cells were grown to log phase of growth and transfected with purified synthesized TAT 72 proteins of wild type, K 50/51 substituted with R, or K 50/51 substituted with A. A total of 3 μg of LTR-CAT DNA along with 0.5 μg of TAT proteins was electroporated into CEM cells (at 230 V). Extracts were analyzed 24 h after transfection for the presence of CAT enzyme.

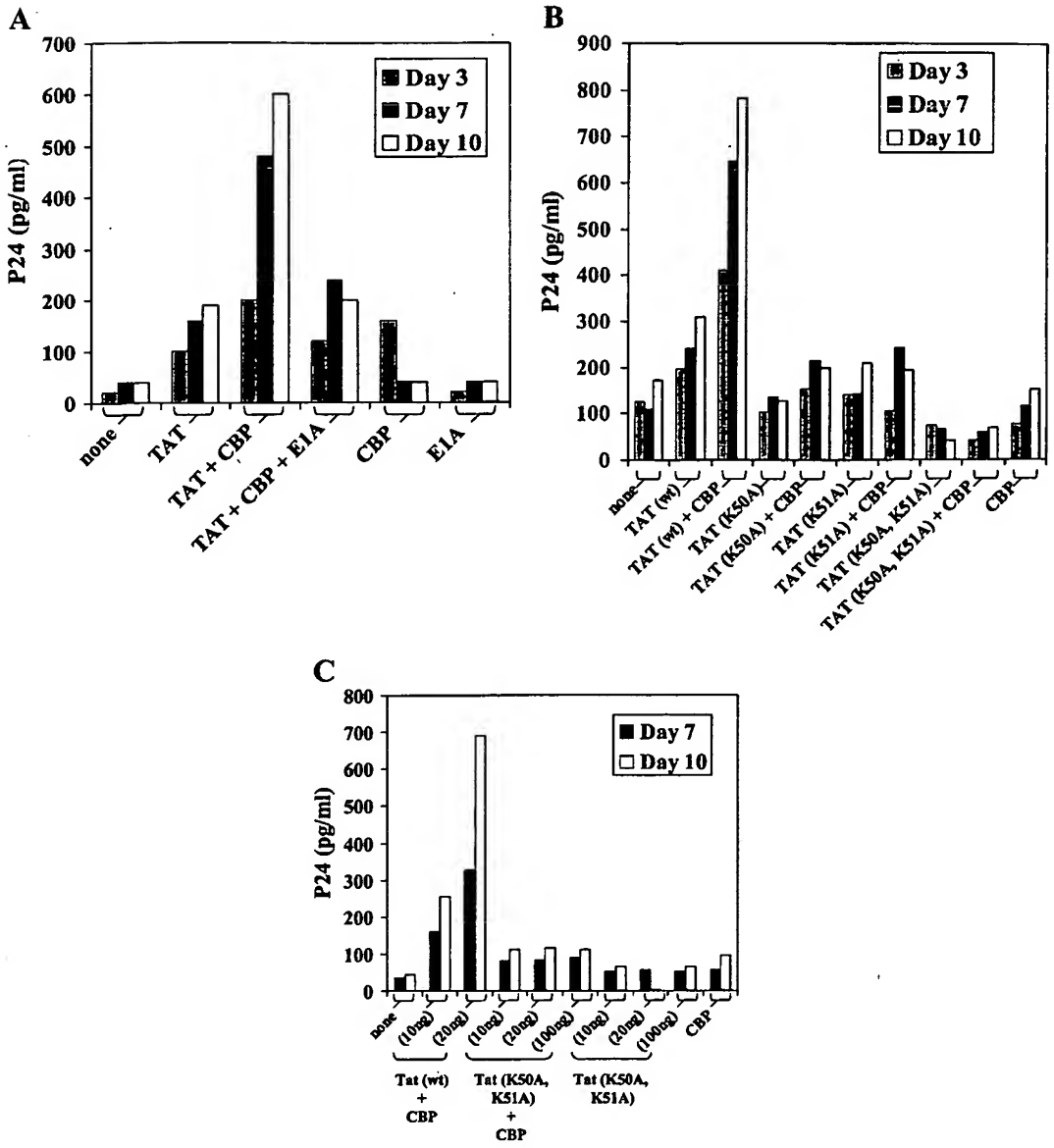


FIG. 6. Both lysines K50 and K51 of Tat play an important role in activation of the proviral integrated HIV-1 DNA. HLM1 cell is a HeLa-derived cell line that contains a wild-type integrated copy of the HIV-1 proviral genome except for the Tat open reading frame. Therefore, HLM1 cells are negative for virus particle production unless they are provided with cytokine signals, such as TNF, or ectopic addition of Tat, as determined by the presence of p24 gag antigen in the supernatant. (A) Coactivation of integrated HIV-1 provirus by Tat and CBP. HLM1 cells were transfected alone with Tat (50 ng), CMV-CBP (6 μg), E1A (6 μg), or in combination with each other by the $CaPO_4$ precipitation method. The p24 gag antigen ELISA was performed from supernatants obtained at days 3, 7, and 10 after transfection. It is interesting to note that cotransfection of Tat plus CBP increased the production of HIV-1 particles in HLM1 cells and E1A was able to reverse the CBP effect, presumably by binding to CBP and inhibiting the HAT activity. (B) HLM1 cells were transfected either alone with wild-type Tat and mutant Tat clones (single-mutants K50A and K51A and double-mutant K50A/K51A) or with CBP into HLM1 cells and p24 gag antigen measured at various time points. (C) Titration of double-mutant Tat (K50A/K51A) with CBP performed in HLM1 cells. Varying concentrations of the Tat double-mutant were transfected either alone or with 6 μg of CBP. Supernatants were collected at days 7 and 10 for the p24 gag ELISA assay. All experiments in A, B, and C were performed twice.

either enhancers or repressors of gene expression during cellular activation. Members of the p300 and CBP family also appear to be present only in higher eukaryotic cells including *Caenorhabditis elegans* and *Drosophila,* and not in the yeast *Saccharomyces cerevisiae.* Thus, p300/CBP-like proteins are likely confined to multicellular organisms where they may fulfill specific functions required for proper growth and development.

Many viruses have evolved mechanisms to control both viral and host transcriptional machinery through CBP/p300. Generic virus infection of cells results in a dramatic hyperacetylation of histones H3 and H4 by CBP/p300, which is localized to target cellular promoters such as IFN-β promoter (Parekh and Maniatis, 1999). Also, both DNA and RNA viruses have evolved means to control CBP/p300 in both activation and repression. For

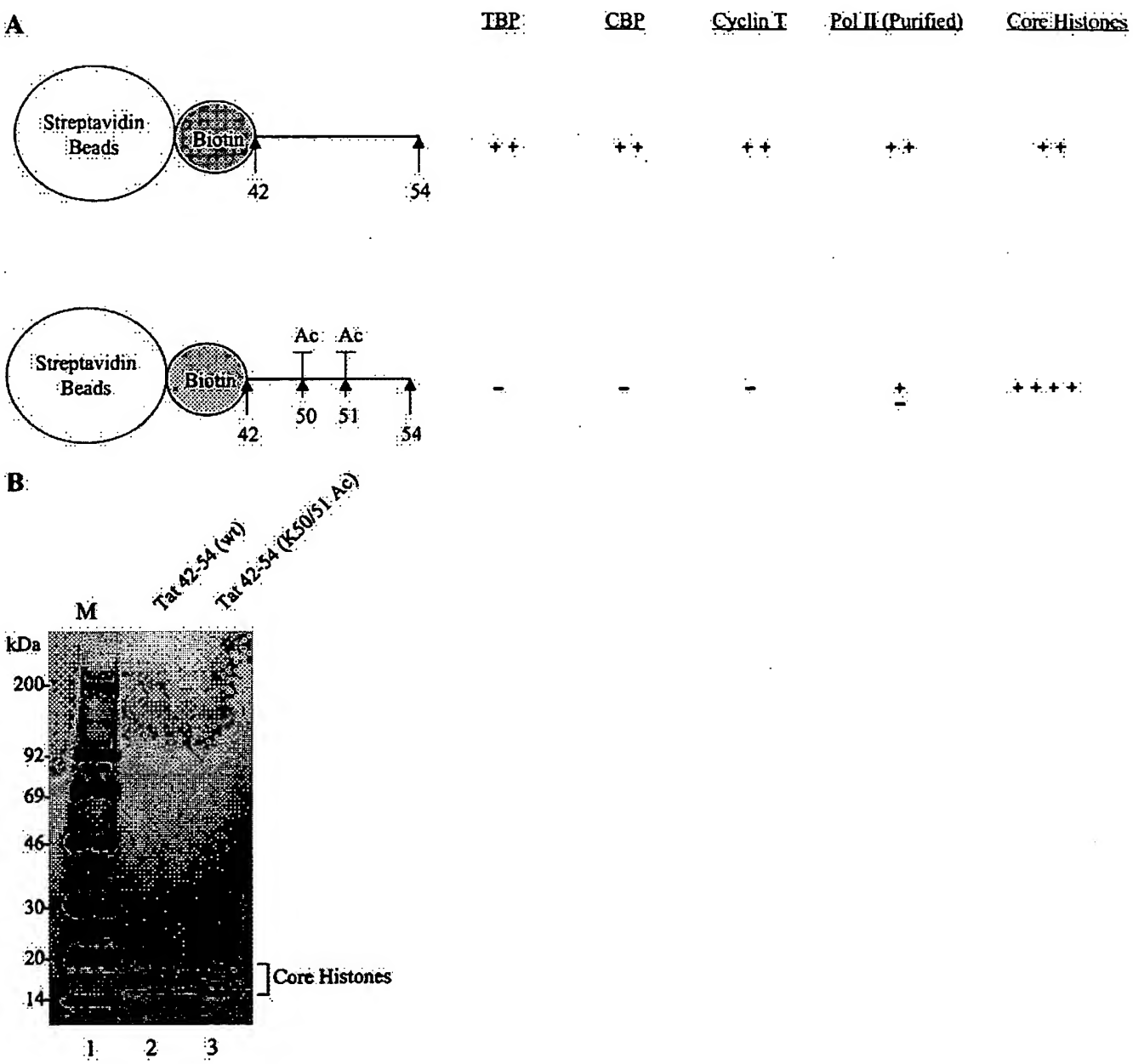


FIG. 7. Binding of acetylated Tat to various transcription factors. (A) Schematic representation of the pull-down experiment and the results of Western blots of the pull-down components with anti-TBP, CBP, cyclin T, and Pol II. The synthesized Tat peptides (42–54) with or without acetylated lysines at positions 50 and 51 were labeled with biotin at the N-terminus. The peptides were incubated with *in vitro* translated, [^{35}S]methionine/cysteine-labeled TBP, CBP, cyclin T, and purified core-HeLa Pol II. Streptavidin–agarose beads were used to pull down the peptide-associated complexes. The pull-down complexes were washed three times and separated on the 4–20% SDS–PAGE gel. For Western blots they were transferred to PVDF membranes and blotted with the antibodies against TBP, CBP, cyclin T, and Pol II. Pull-down experiments with ^{35}S-labeled TBP, CBP, and cyclin T were also performed to confirm the Western blot data. (B) Pull-down complexes from chromatin assembled HIV-1 DNA with the wild-type unacetylated Tat peptide (42–54, lane 2) or double-acetylated Tat peptide (42–54) at positions 50 and 51 lysines (lane 3) were separated on SDS–PAGE, stained with Coomassie blue, excised, subjected to digestion for trypsin digest, and analyzed by mass spectrometry. Western blots for histones H2A, H2B, H3, and H4 were also performed to confirm the mass spectrometry analysis (data not shown).

instance, the oncogenic human papillomavirus type 16 (HPV-16) E6 binds to three regions (C/H1, C/H3, and the C-terminus) of both CBP and p300. Interestingly, HPV-16 E6 inhibits the intrinsic transcriptional activity of CBP/p300 and decreases the ability of p300 to activate p53- and NF-κB-responsive promoter elements (Patel *et al.*, 1999). Similarly, human herpesvirus 8/Kaposi sarcoma-associated virus IRF 1 protein also targets the carboxy-terminal region (aa 1623 to 2414) of the transcriptional coactivator p300 (Burysek *et al.*, 1999). RNA retroviruses such as MMTV, HTLV-I, and HIV have also been noted for regulating p300/CBP. CBP suppresses the responsiveness of the mouse mammary tumor virus (MMTV) promoter to dexamethasone in a dose-dependent fashion

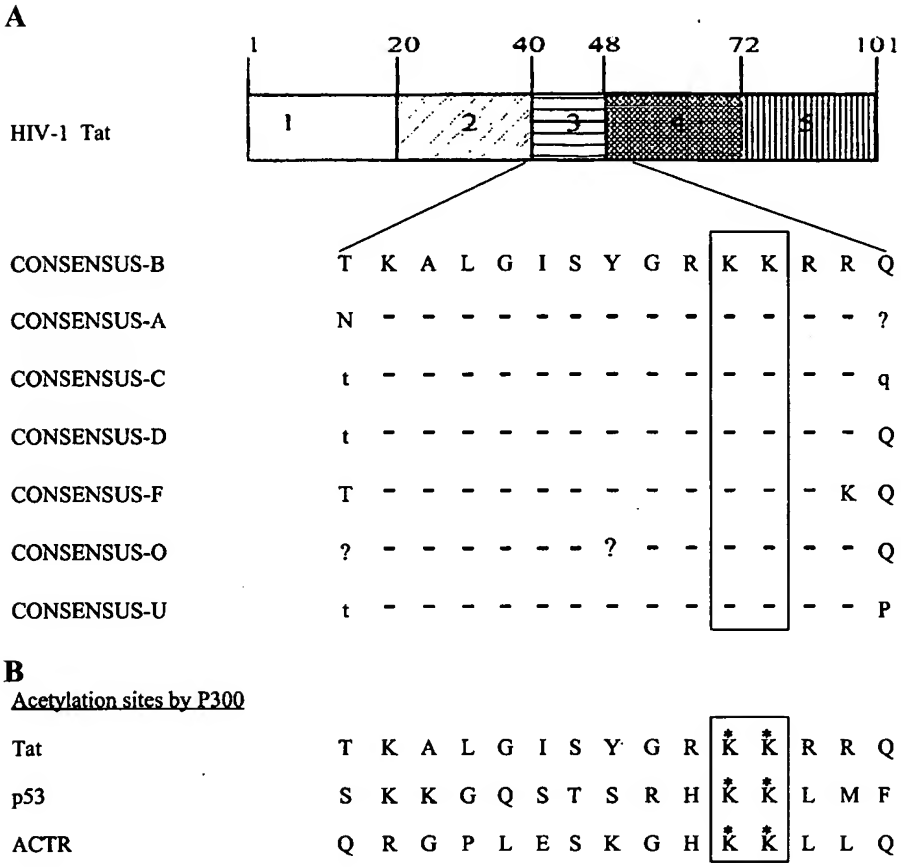


FIG. 8. A general scheme of various Tat domains. (A) Conservation of the double-lysine motif of Tat among various HIV-1 clades. The Tat protein and peptides used in this study were of North American Clade B type. (B) The double-lysine motifs of Tat resemble those in p53 and ACTR proteins, which are also acetylated by CBP/p300 as indicated by asterisks.

(Kino *et al.*, 1999), as well as alleviating NFI-C-mediated repression of MMTV by ectopic expression of p300/CBP (Chaudhry *et al.*, 1999). Transcriptional activation of the HTLV-I sequences by Tax and CBP is induced by reinitiation of transcription (Kashanchi *et al.*, 1998), and cytokine and Tat regulation of HIV transcription requires binding of the p300 coactivator to the promoter region (Hottiger *et al.*, 1998; Hottiger and Nabel, 1998; Benkirane *et al.*, 1998; Marzio *et al.*, 1998; Kiernan *et al.*, 1999; Ott *et al.*, 1999).

CBP/p300 acetylates the core histones as well as nonhistone proteins such as the tumor suppressor protein p53, the hematopoietic transcription factor GATA-1, and the basal transcription factors TFIIE and TFIIF, although their biological functions are still not well understood. To test whether HIV-1 Tat could also be specifically acetylated by CBP/p300, we used the purified full-length Tat (1–86) protein and found that the labeling was completely dependent on the presence of the CBP/p300 HAT domain. The labeling was most efficient with purified Tat and not GST–Tat (1–101), GST–Tat (1–86), GST–Tat (1–72), or 17 other GST–Tat mutants.

When using Tat peptides to pinpoint the amino acid residues important for acetylation, we found that the both lysines in the highly conserved region (^{49}RKKRRQ^{54}) of the basic RNA-binding motif of Tat were acetylated by CBP/p300 (see Fig. 8). Importantly, upon transfection of K50A or K51A Tat vectors with reporter HIV-LTR CAT, we observed a slight drop in transcriptional activity but a more pronounced drop in transactivation with the double-mutant K50A and K51A. This suggested that both lysines together are important in the transient transfection assays. However, when using HLM1 cells, containing integrated virus, we observed that both point mutations at position 50 and 51 were equally deleterious in activation of the integrated chromatinized HIV-1 DNA and that ectopic expression of the CBP could not activate either of the 50 or 51 mutants in these cells. Therefore, we believe that both lysines are equally important for *in vivo* activation of the latent virus.

When examining for the effect of wild-type or acetylated Tat on RNA binding, we found that the acetylated Tat peptide or protein was not able to bind to TAR RNA. This is in marked contrast to other acetylated proteins such as p53, where acetylation increases the DNA-binding activity (Gu and Roeder, 1997). More interestingly, when performing competition experiments with wild-type, acetylated, or alanine-substituted Tat peptides in

the same reaction, we observed that wild-type 41–54 but not other derivatives was able to enhance the binding of CBP to Tat as well as other basal factors such as TFIIB and TBP. This intriguing result indicates that CBP/p300 family members are subject to conformational change upon binding to viral and possibly cellular activators. In support of the change of conformation hypothesis, we have recently obtained preliminary data suggesting that *in vitro* translated CBP in presence of wild-type but not acetylated Tat peptide is susceptible to endoproteinase Glu-C digestion and not to other nucleases such as trypsin, endonuclease Asp-N, or Lys-C proteases.

CBP/p300's ability to acetylate Tat has recently been examined in a chromatin reconstitution experiment. When using purified basal transcription factors NF-κB, SP1, and cdk9/cyclin T in an *in vitro* transcription reaction, where the HIV-1 DNA is chromatinized, we observed no activated transcription *in vitro,* indicating that the mere presence of cdk9/cyclinT is not sufficient to drive RNA pol II through chromatin DNA. However, only in the presence of added minimal p300 HAT domain or wild-type p300 do we find activated transcription on HIV-1 DNA (Deng, unpublished data). Therefore, the role of the cdk9/cyclinT complex in HIV-1 transcription may be to phosphorylate the CTD of RNA pol II and that of p300 may be to acetylate the natural core histones on the HIV-1 genome. To this end, we have mapped the promoter region (−500 to +200) of 26 different HIV-1 clade isolates ranging from subtypes B to O and have observed that all viral isolates have chromatinized DNA *in vivo,* further indicating that the HIV-1 B clade that we have used in this study (in HLM1 cells) was not the only HIV-1 chromatinized template *in vivo.* Finally, our results show that the acetylated Tat decreases Tat's ability to bind the TAR RNA element, as well as to bind basal factors TBP and core-Pol II, but increases the efficiency of binding to core histones and only when assembled as a nucleosomal HIV-1 DNA. This notion may provide a mechanism of how Tat is able to leave the initiation complex behind and to facilitate chromatin modification or remodeling downstream of the transcription initiation site, perhaps by aiding in disruption of the nuc-1 nucleosome. El Kharroubi *et al.* (1998) demonstrated that expression of a functional Tat could alter the chromatin structure downstream of the HIV-1 promoter and that the binding of Tat to TAR (as occurs with Tat K41T) failed to induce chromatin remodeling. Recently, using *in vitro* reconstituted chromatin templates, we found that Tat–p300 interaction increases the acetylation of a nucleosomal histone. Such enhancement of histone acetylation may be due to the finding that the acetylated Tat binds with higher efficiency to nucleosomal DNA and changes the conformation, resulting in the accessibility of histone tails to p300. Acetylation of histones may flag other proteins needed for disruption of nuc-1 and subsequent transcription. Future experiments will determine the nucleosomal position(s) that is affected by acetylated Tat on whole HIV-1 DNA.

MATERIALS AND METHODS

Cell culture and labeling

Log-phase CEM (12D7) T-lymphocytes (Kashanchi *et al.,* 1992, 1994a, 1994b) were grown in culture at 37°C up to 1×10^5 cells/ml in RPMI 1640 medium containing 10% fetal bovine serum treated with a mixture of 1% streptomycin, penicillin, and 1% L-Glutamine (Gibco BRL). HLM-1 cells (AIDS Research and Reference Reagent Program, Catalog No. 2029) were derived from HeLa-T4$^+$ cells integrated with one copy of the HIV-1 genome containing a Tat-defective mutation. The mutation was introduced as a triple termination linker at the first AUG of the Tat gene (Sadaie and Hager, 1994). HLM-1 cells are negative for virus particle production, but can be induced to express noninfectious HIV-1 particles after transfection with Tat cDNA or mitogens such as TNF-α or sodium butyrate. HLM1 cells were grown in Dulbecco's modified Eagle's medium (DMEM) containing 100 μg/ml of G418, plus 1% streptomycin, penicillin, and 1% L-glutamine (Gibco BRL). These cells were always grown to 75% confluency before transfection or passages.

Labeling experiments were performed on log-phase 75% confluent HeLa/eTat or control HeLa/pcep4 cells (Kashanchi *et al.,* 2000). Cells were pulsed for 3 h with [^{3}H]acetate (0.4 mCi, ICN) in complete DMEM plus hygromycin. Cells were also incubated overnight with [^{35}S]methionine/cysteine (0.2 mCi/ml, NEN) in methionine/cysteine-free medium and 10% dialyzed FCS. Nuclear lysates were prepared in 1 ml of lysis buffer (300 mM NaCl, 0.1% NP-40, 10 mM NaH_2PO_4, 1 mM EDTA, pH 8, 1 mM DTT, 50 mM sodium pyrophosphate, 10 mM NaF, 8 mM sodium butrate), and immunoprecipitations were performed on 0.5 mg protein extract. Tat was detected using immunoprecipitation from cellular lysates with monoclonal 12CA5 Ab cross-linked to beads for 3 h and eluted with a 100-fold excess of influenza peptide for 8 h at 4°C. The sequence of peptide used for elution was as follows: ^{N}YPYDVPDYASL^C. Four independent [^{3}H]acetate and [^{35}S]methionine/cysteine labeling experiments were performed with similar results.

Lymphocyte transfection

Lymphocytes (CEM, 12D7) were grown to mid-log phase of growth and were processed for electroporation according to a previously published procedure (Kashanchi *et al.,* 1992). Only one modification was introduced, where cells were electroporated at 230 V and plated in 10 ml of complete RPMI 1640 medium for 18 h prior to harvest and CAT assay.

Transfection and HIV-1 detection of HLM_1 cells

HLM_1 cells were propagated in DMEM (containing 100 μg/ml of G418) and transfected with the plasmid DNAs including Tat, mutant Tat, and CBP using the calcium phosphate method. The transfected cells were washed after 4 h and fresh complete DMEM with 10% fetal bovine was added for the remainder of the experiment. The p24 gag antigen was detected with a standard ELISA kit (Abbott) using the supernatants of transfected cells at days 3, 7, and 10.

Plasmids

HIV-LTR-CAT reporter and eukaryotic Tat expression vectors (pcTat) have been described previously (Hauber *et al.*, 1989; Kashanchi *et al.*, 1994b). Mutants of the lysine residue at position 50 and/or 51 of the Tat expression plasmid were constructed from pcTat, by replacing the *Eco*NI fragment with synthesized mutated oligo adaptor. The following top strands for each mutated construct are indicated: for K50A, 5′GGCAGGGCGAAGCGGAGACAGCGACGAAGACCTCC3′; for K51A, 5′GGCAGGAAGGCGCGGAGACAGCGACGAAGACCTCC3′; and for K50/A+K51/A, 5′GGCAGGGCGGCGCGGAGACAGCGACGAAGACCTCC3′. Eukaryotic expression vectors for CBP E1A have previously been reported (Chakravarti *et al.*, 1999). GST–p300 (HAT) from aa 1197 to 1735 was PCR amplified from a p300 B fragment as well as the p300 C fragment and subcloned into pGEX (more details of subcloning will be provided upon request). The resulting recombinant vector was transformed into *E. coli* DH5a and were grown overnight in 10 ml of LB with 100 μg/ml of ampicillin. A 500-ml LB + ampicillin flask was inoculated with the overnight culture and was grown for 4 h at 37°C. Isopropyl-1-thio-*b*-D-galactopyranoside was added to a final concentration of 0.1 mM to induce fusion protein expression, and the culture was switched to 30°C for an additional 4 h. Cells were collected by centrifugation in a GSA rotor at 5800 *g* for 10 min at 4°C. For sonication, the bacterial pellet was resuspended in 25 ml of phosphate-buffered saline containing 1 mM phenylmethylsulfonyl fluoride and was sonicated (Branson) for 35 pulses at the 3.5 microprobe setting. The resulting mixture was centrifuged in a GSA rotor at 5800 *g* for 10 min at 4°C. A second centrifugation in a SS-34 rotor at 23,500 *g* for 20 min at 4°C clarified the extract of remaining debris. GST–fusion proteins were bound to agarose beads overnight, washed the next day, and run on 4–20% SDS–PAGE for both quality and quantity prior to use in HAT assays.

Baculovirus expression and protein purifications

Sf9 cells were grown to mid-log phase in HyClone HyQ CCM-3 serum-free medium utilizing spinner flask culture methods. For p300 infection, the cells were infected with 11 ml of p300 FLAG virus at 2.0×10^6 cells/ml and then allowed to incubate at 10 rpm for approximately 1 h. After this initial incubation/infection time the spinner plates were turned up to 70 rpm for the remainder of the incubation. The cells were collected via centrifugation 48 h after infection. The cell culture normally yielded approximately 2 ml of PCV from 500 ml of original culture volume, and the cell pellet was processed for further purification. Samples were lysed with lysis buffer, containing 50 mM Tris–Cl, pH 7.4, 120 mM NaCl, 5 mM EDTA, 0.5% NP-40, 50 mM NaF (phosphotyrosine phosphatase inhibitor), 1 mM DTT, and 1 mM PMSF, and processed with 12CA5 monoclonal antibodies for further purifications.

Histone acetyltransferase assay

HAT assays were performed as 30-μl total reactions at 37°C for 60 min in buffer containing 1 μl of purified GST–p300 HAT (1 mg/ml), 200–400 ng substrate proteins or peptides, and 1 μg of histones H2A, H2B, H3, and H4 in 20 mM HEPES–NaOH, pH 7.4, 1 mM dithiothreitol, 10 mM sodium butyric acid, and 1 μl of [^{14}C]acetyl-Coenzyme A (65 mCi/mmol, ICN). Proteins and peptides were resolved on 4–20% or 15% SDS–PAGE. Gels were dried and exposed to a PhosphoImager cassette.

Preparation of TAR RNA and RNA-binding experiments

The plasmid pT7 was constructed from pU3R-III containing a T7 promoter at nucleotide +1 of HIV (Gunnery *et al.*, 1990). pT7 was linearized at nucleotide +82 by digestion with *Hin*dIII and transcribed using T7 RNA polymerase (Promega). TAR RNA was labeled with [α-^{32}P]UTP and was subjected to electrophoresis in a 10% polyacrylamide gel. The major radioactive RNA band was eluted and extracted with phenol/chloroform and precipitated with ethanol.

Gel mobility shift reaction (16-μl final reaction volume) was carried out in binding buffer (10 mM HEPES, pH 7.3; 100 mM KCl; 1 mM $MgCl_2$; 0.5 mM EDTA; 1 mM DTT; and 10% glycerol) and contained 3 ng of labeled TAR RNA as well as 200 ng tRNA as nonspecific competitor. Reactions were incubated for 30 min at room temperature and RNA-bound complexes were separated on a prerun 6% DNA retardation gel (Novex) containing 0.5× TBE buffer, at 7 W for 2.5 h at 4°C.

Peptide synthesis

The biotinylated peptides were prepared on a PAL–PEG–polystyrene resin by continuous-flow solid-phase synthesis on a PerSeptive Biosystems Pioneer synthesizer (Framingham, MA) using HBTU-activated Fmoc amino acids. Side chain protection was as follows: Arg (Pmc), Gln (Trt), Lys (Dde), Ser, and Tyr (Bu^t). Peptide assembly was concluded by N$^\alpha$-acylation with HBTU-activated biotin. The resin-bound peptide was then

treated with 3% hydrazine in DMF for 20 min to selectively remove the Dde groups from the side chain of Lys. The resin was then divided into two equal portions and one-half was subjected to a 1-h treatment with an excess of acetic anhydride in the presence of an equivalent amount of base to acetylate the resulting free N^{ϵ} groups. Both peptides were then separately cleaved from the solid support and simultaneously the remaining side chain was deprotected by reaction with trifluoroacetic acid in the presence of scavengers. Peptide purification was achieved by conventional reverse-phase HPLC on Vydac C18 (Hesperia, CA) in an overall yield of 25–30% based on starting resins. The purity of the two peptides was confirmed by analytical reverse-phase HPLC, capillary zone electrophoresis, and matrix-assisted laser desorption time of flight mass spectrometry. For the non-acetylated peptide, we found MH^+ 1757.5 (calc. MH^+ 1757.1). For the acetylated peptide, we found MH^+ 1840.9 (calc. MH^+ 1840.1).

Synthesis of acetylated peptides at positions 50, 51, and 50+51 was carried out on the ABI 433A Peptide Synthesizer (PE Biosystems, Foster City, CA) using Fast-moc chemistry with N^{ϵ}-acetyl-L-lysine, which was purchased from Novabiochem (San Diego, CA). After cleavage and deprotection, the peptides were purified by HPLC (Dionex, Sunnyvale, CA) using an acetonitrile gradient on a C18 reverse-phase column (Pharmacia, Piscataway, NJ). The amount of protein was determined by Bio-Rad protein assay as well as by running small aliquots on 4–20% SDS–PAGE followed by silver staining.

Streptavidin bead pull-down assay

Synthesized Tat (42–54) peptides, labeled with biotin at the N-terminus, and with or without an acetyl group at lysines 50 and 51, were used in the pull-down assays. The biotin-labeled Tat peptides were incubated with the cell extracts in TNE_{50} buffer (100 mM Tris–HCl, pH 7.5; 50 mM NaCl; 1 mM EDTA; 0.1% NP 40) at 4°C overnight. Streptavidin beads (Boehringer Mannheim) were added to the mixture and incubated for 2 h at 4°C. The beads were washed three times with TNE_{150} (100 mM Tris–HCl, pH 7.5, 150 mM NaCl; 1 mM EDTA; 0.1% NP-40). The bound proteins were separated on 4–20% SDS–PAGE and subjected to Western blotting with antibodies against TBP, CBP, cyclin T, and RNA polymerase II (Santa Cruz Inc.; SC-900 (C21) for Pol II, Sc-1211 (451) for CBP, Sc-204 (N12) for TBP, and cyclin T (a generous gift from M. Mathews).

Nucleosome reconstitution by salt dialysis

The core histones were purified from HeLa cells by the method of Simon and Felsenfeld (1979). Chromatins were prepared from high-molecular-weight DNA and plain and purified core histones by dialysis from 1 M NaCl (Imbalzano, 1998; Stein, 1989). Ten micrograms of plasmid DNA of pDH125 (whole HIV-1 genome, a generous gift from M. Cho and M. Martin, NIAID/NIH) was mixed with 5 μl of 5 M NaCl and 2 μl of 10× reconstitution buffer (0.15 M Tris–HCl, pH 7.5; 1 mM DTT; 2 mM EDTA) by pipetting up and down repeatedly. Later, 15 μg of core histones was added in a total volume of 20 μl; the volume was adjusted by adding ddH_2O. Samples were gently flicked in the tube to mix and incubated at 37°C for 20 min. Sequential dilution was carried out by adding 10 μl of 1× reconstitution buffer every 10 min, for 3 h at 37°C. At each time point, samples were mixed by pipetting up and down. An aliquot was run on agarose gels to ensure proper assembly prior to each experiment.

In-gel digestion, mass spectrometry, and protein identification

The in-gel digestion was performed based on a procedure previously described by Fernandez *et al.* (1998). The gel bands of interest were excised from SDS–PAGE and digested with 0.2 μg of trypsin (Promega modified sequencing grade trypsin). The digests were desalted using C_{18} ZipTips (Millipore) according to the manufacturer's protocol. A 1-μl aliquot of sample was taken for peptide mass mapping on a PerSeptive Biosystem DEPRO MALDI–TOF Mass Spectrometer using α-cyano-4-hydroxycinnamic acid as the matrix. Analysis was performed in the linear delayed-extraction mode, with external calibration. Protein identification by mass mapping was performed through the ProFound Web site located at Rockefeller University (prowl.rockefeller.edu).

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Structural Basis of Lysine-Acetylated HIV-1 Tat Recognition by PCAF Bromodomain

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Summary

The human immunodeficiency virus type 1 (HIV-1) *trans*-activator protein Tat stimulates transcription of the integrated HIV-1 genome and promotes viral replication in infected cells. Tat transactivation activity is dependent on lysine acetylation and its association with nuclear histone acetyltransferases p300/CBP (CREB binding protein) and p300/CBP-associated factor (PCAF). Here, we show that the bromodomain of PCAF binds specifically to HIV-1 Tat acetylated at lysine 50 and that this interaction competes effectively against HIV-1 TAR RNA binding to the lysine-acetylated Tat. The three-dimensional solution structure of the PCAF bromodomain in complex with a lysine 50-acetylated Tat peptide together with biochemical analyses provides the structural basis for the specificity of this molecular recognition and reveals insights into the differences in ligand selectivity of bromodomains.

Introduction

The human immunodeficiency virus type 1 (HIV-1) protein Tat is an atypical *trans*-activator of transcription which functions through binding to an RNA element known as the transactivation responsive region (TAR), located in the retroviral long-terminal repeat (LTR) (Cullen, 1998; Jeang et al., 1999; Karn, 1999). Tat binds to TAR RNA with high affinity but transiently (Keen et al., 1997; Rana and Jeang, 1999). Dissociation of Tat from TAR RNA facilitates Tat association with the assembled RNA polymerase II (RNAPII) complex (Deng et al., 2000; Kiernan et al., 1999). The latter process enables the transcriptional machinery complex to elongate efficiently on the viral DNA template in order to produce full-length HIV transcripts during viral productive replication in infected cells (Adams et al., 1994; Garber and Jones, 1999).

While the detailed molecular mechanisms underlying Tat dissociation from TAR RNA and its transactivation of transcription of the integrated HIV-1 genome remain elusive, increasing lines of evidence suggest that Tat activity requires its association with several multiprotein complexes, which include the cyclinT1/cyclin-dependent kinase 9 (CDK9) complex (Jones, 1997; Wei et al., 1998) and histone acetyltransferase (HAT) transcriptional coactivators, p300/CBP (CREB binding protein), and p300/CBP-associated factor (PCAF) (Benkirane et al., 1998; Deng et al., 2000; Hottiger and Nabel, 1998). Recruitment of CDK9 through the N-terminal cysteine-rich region of Tat results in hyperphosphorylation of the C-terminal domain of RNAPII that increases elongation efficiency of HIV-1 transcription (Wei et al., 1998). Recently, it has been shown that Tat activity is dependent on acetylation by p300/CBP at K50 located in the C-terminal arginine-rich motif (ARM) (Kiernan et al., 1999; Ott et al., 1999), a region that is also important for TAR RNA binding and nuclear localization. Mutation of K50 to arginine, a conserved amino acid substitution that retains the positive charge but prevents acetylation by p300, markedly decreases the synergistic activation of the HIV-1 promoter by Tat and p300 (Kiernan et al., 1999; Ott et al., 1999). Tat acetylation at K50 results in its dissociation from TAR RNA and promotes formation of a multiprotein complex comprised of Tat, p300/CBP, and PCAF (Benkirane et al., 1998; Deng et al., 2000). Furthermore, it has been shown that the HAT activity of PCAF is preferentially required for Tat transactivation of transcription of the integrated but not the unintegrated HIV-1 LTRs (Benkirane et al., 1998).

Protein lysine acetylation is emerging as a central mechanism in regulation of chromatin remodeling and transcriptional activation (Jenuwein and Allis, 2001; Kouzarides, 2000; Strahl and Allis, 2000). Bromodomains, an extensive family of conserved protein modules found in many chromatin-associated proteins and in nearly all nuclear histone acetyltransferases (Brownell and Allis, 1996; Haynes et al., 1992; Jeanmougin et al., 1997; Tamkun et al., 1992), have been recently discovered to function as acetyl-lysine binding domains (Dhalluin et al., 1999; Hudson et al., 2000; Jacobson et al., 2000; Owen et al., 2000). This new finding suggests a novel mechanism for regulating protein-protein interactions via lysine acetylation (Dyson et al., 2001; Jenuwein and Allis, 2001; Strahl and Allis, 2000; Winston and Allis, 1999). This new mechanism supports the hypothesis that bromodomains could contribute to highly specific histone acetylation by tethering transcriptional HATs to specific chromosomal sites (Brownell and Allis, 1996; Manning et al., 2001; Travers, 1999), and to the assembly and activity of multiprotein complexes of chromatin remodeling such as SAGA and NuA4 (Brown et al., 2001; Sterner et al., 1999). However, because no specific, biologically relevant binding sites had been reported for any particular bromodomain, the major question of ligand specificity of bromodomains still remains unanswered.

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Ac-Tat 102(a)/(b) PCAF ?

In efforts to determine the mechanisms of action of Tat in transactivation of HIV-1 transcription, we investigated whether the interaction of the activated, lysine-acetylated Tat with the nuclear HAT transcriptional cofactors p300/CBP and PCAF involves any of the bromodomains of the latter proteins. Here, we report that the bromodomain of PCAF but not CBP can specifically recognize the lysine-acetylated Tat at K50 (not K51 or K28), and this interaction competes effectively against TAR RNA binding to the acetylated Tat. We have also determined the three-dimensional structure of the PCAF bromodomain in complex with a lysine-acetylated peptide derived from Tat at K50 by using nuclear magnetic resonance (NMR) spectroscopy. NMR structural and biochemical analyses were further used to gain structural insights into this important molecular recognition as well as the differences in ligand selectivity of different bromodomains.

Results and Discussion

PCAF Bromodomain Recognition of Lysine-Acetylated HIV-1 Tat at K50

To test whether Tat-p300/CBP-PCAF association involves bromodomains of the histone acetyltransferase transcriptional coactivators, we performed an in vitro binding assay by using recombinant and purified GST-fusion bromodomains and lysine-acetylated peptides derived from known acetylation sites in Tat at K28 and K50. A lysine-acetylated Tat peptide containing AcK50 (SYGR-AcK-KRRQR, where AcK is an N^{ϵ}-acetyl-lysine) showed no detectable interactions with either bromodomains or bromodomain and PHD finger (Aasland et al., 1995) tandem modules from CBP or TIF1β (transcriptional intermediary factor 1β, also named KAP-1 and KRIP-1) (Friedman et al., 1996) (Figure 1A). Strikingly, the same Tat peptide bound tightly to the bromodomain of PCAF, which shares high sequence homology to CBP bromodomain (Jeanmougin et al., 1997). The binding is dependent on acetylation of K50 on Tat. Neither of these bromodomains interacted with an acetylated Tat peptide derived from K28 (TNCYCK-AcK-CCFH) (data not shown), highlighting the selective nature of PCAF bromodomain recognition of the Tat AcK50 sequence.

We performed an NMR study in order to determine the specificity of PCAF bromodomain binding to lysine-acetylated Tat. As anticipated, PCAF bromodomain did not bind to Tat AcK28 peptide, nor did CBP bromodomain to either Tat AcK28 or AcK50 peptide. In contrast, PCAF bromodomain bound to Tat AcK50 peptide with high affinity and caused extensive chemical shift perturbations of protein amide resonances, significantly greater than those seen with an acetylated peptide derived from histone H4 at K16, as shown in 2D ^{1}H-^{15}N heteronuclear single quantum coherence (HSQC) spectra (Figure 1B). This observation agrees with the differences in dissociation constants (K_D), determined by NMR titration to be ~10 μM and >300 μM for the former and latter complexes, respectively. These results argue that PCAF bromodomain binding to H4 peptide is largely limited to the acetyl-lysine, whereas its recognition of Tat most likely involves additional interactions with residues flanking AcK50.

To assess the biological relevance of the PCAF bromodomain and Tat interaction to the activation of Tat transcriptional activity by PCAF and p300/CBP (Benkirane et al., 1998; Kiernan et al., 1999), we performed cell transfection experiments and measured their combined effect on the activity of the HIV-1 promoter using an HIV-1 LTR-luciferase reporter gene assay (Bieniasz et al., 1998; Madore and Cullen, 1993). As shown in Figure 1C, synergistic activation of Tat-mediated transcription of the HIV-1 promoter in human 293T cells is dependent upon both PCAF and CBP. The latter HAT transcriptional coactivator has been recently shown to be responsible for lysine acetylation of Tat at K50 that is required for Tat activation (Kiernan et al., 1999; Ott et al., 1999). More importantly, our data show that cotransfection of the PCAF bromodomain but not the CBP bromodomain resulted in a significant reduction of the synergistic activation of Tat by PCAF and CBP, likely due to an effective competition of the PCAF bromodomain against the full-length PCAF binding to Tat. Collectively, our in vivo transfection study further confirms the highly specific nature of PCAF bromodomain/Tat recognition and highlights the functional importance of this bromodomain interaction in the synergistic PCAF- and CBP-induced activation of Tat transcriptional activity in HIV-1 gene expression.

Structure of the PCAF Bromodomain/Tat Peptide Complex

To understand the structural basis of this molecular recognition, we determined the three-dimensional structure of the PCAF bromodomain in complex with Tat AcK50 peptide from a total of 2903 NMR-derived restraints. The structure for the protein (residues 723–830) and the peptide (residues 47–54) complex was well defined by the NMR data (Figure 2A, Table 1). The structure of the bromodomain when complexed to the Tat peptide consists of a left-handed four-helix bundle (helices α_Z, α_A, α_B, and α_C) and is similar to its free form structure (Dhalluin et al., 1999), except for the ZA and BC loops that comprise the acetyl-lysine binding site and undergo local conformational changes to accommodate peptide binding (see below). The Tat peptide adopts an extended conformation and lies across a pocket formed between the ZA and BC loops (Figure 2B). The side chain of the acetyl-lysine intercalates into the protein hydrophobic cavity and interacts extensively with residues F748, V752, Y760, I764, Y802, and Y809 (Figure 2C). Peptide residues flanking the acetyl-lysine contact the protein. Particularly, G(AcK−2), R(AcK−1), and R(AcK+3) showed intermolecular NOEs to the protein, and extensive interactions were observed between the side chains of Y(AcK−3) and V763 and between Q(AcK+4) and E756. These specific interactions confer a highly selective association between PCAF bromodomain and Tat.

Structural comparison of PCAF bromomdomain in the free and ligand-bound forms reveals that structural changes of the protein, largely localized in the ZA and BC loops, are directly coupled with the peptide binding (Figure 2D). These structural changes are supported by extensive NMR data, which include changes of chemical shifts and NOE patterns for the backbone amides, side chain methyl groups, and aromatic rings of many protein

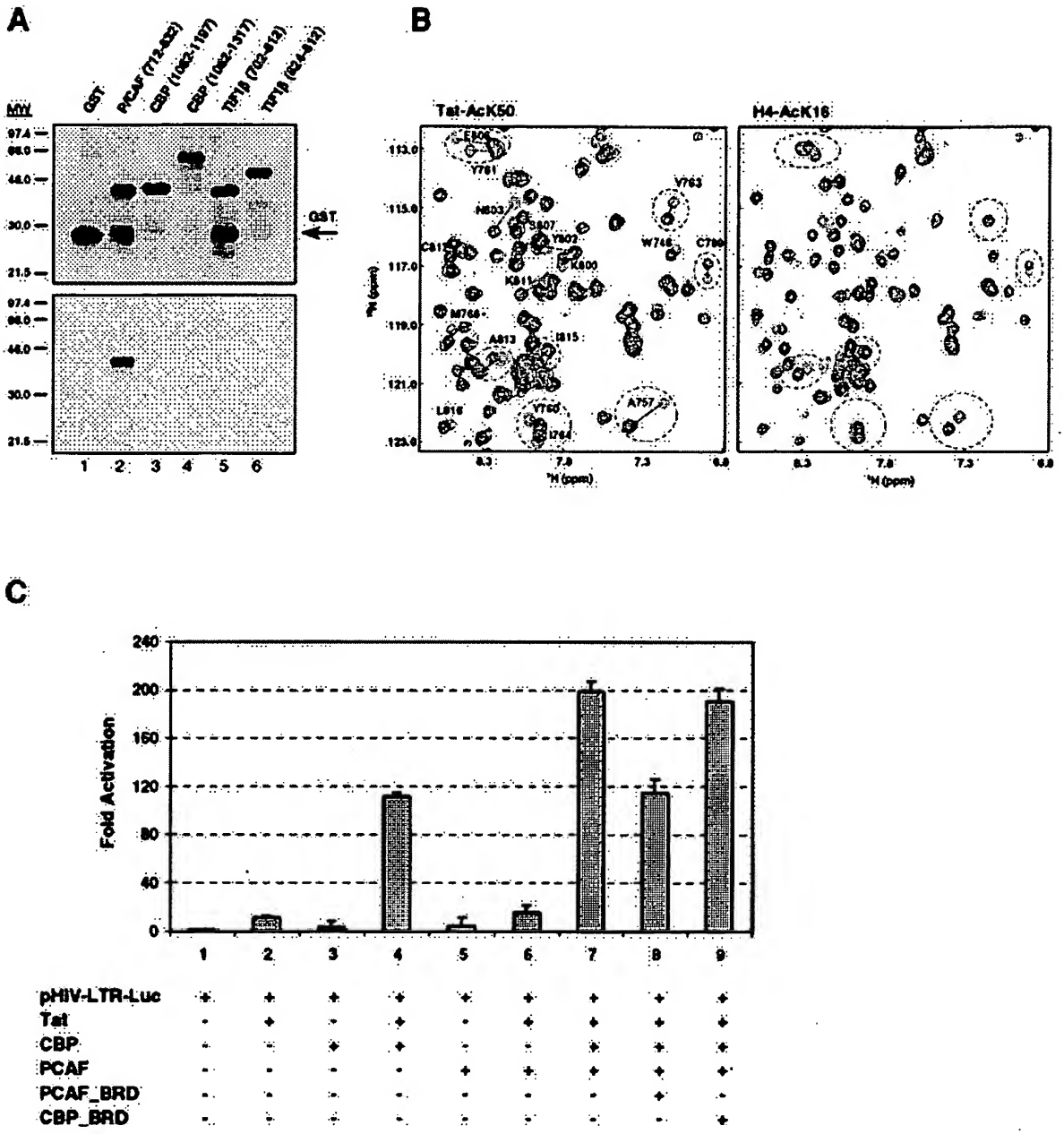


Figure 1. Recognition of Lysine-Acetylated HIV-1 Tat by PCAF Bromodomain

(A) Binding of bromodomains alone from PCAF, CBP, and TIF1β or in combination with PHD fingers from CBP and TIF1β to a biotinylated and lysine-acetylated Tat AcK50 peptide immobilized on streptavidin agarose beads. The upper panel shows purity of the GST-fusion bromodomains used in the assay, separated by SDS-PAGE and stained with Coomassie blue. The lower panel depicts Western blot with anti-GST antibodies, showing specific interaction between PCAF bromodomain and Tat AcK50 peptide.

(B) Comparison of PCAF bromodomain binding to lysine-acetylated peptide derived from HIV-1 Tat at K50 (SYGR-AcK-KRRQR) (left) versus one derived from histone H4 at K16 (SGRGKGGKGLGKGGA-AcK-RHRK) (right). The protein samples were completely titrated with the lysine-acetylated peptide of Tat or H4 with molar ratio 1:1.5 or 1:6, respectively. The 2D ^{1}H-^{15}N HSQC spectra of the bromodomain show changes of backbone amide resonances of the protein in the presence (red) or absence (black) of the peptide ligand. Blue dashed cycles highlight protein residues that exhibited major chemical shift perturbations upon Tat AcK50 peptide binding (left), significantly greater than those observed upon addition of the histone H4 peptide (right).

(C) Functional contribution of PCAF bromodomain and Tat interaction to synergistic stimulation of Tat transcriptional activity by PCAF and CBP. The plasmids used in various combinations in transfections with human 293T cells are as indicated below the graph. The amounts of the plasmids used in transfection experiments are pHIV-LTR-Luc (100 ng), pcTat (100 ng), pRSV-HA-CBP (2.0 ug), pCI-FLAG-PCAF (2.0 ug), pCMV-HA-PCAF_BRD (0.5 ug), and pCMV-FLAG-CBP_BRD (0.5 ug). Total amounts of DNA for transfections were kept constant with addition of empty control vector. Luciferase activities of the cell cytoplasmic extracts were measured using a luciferase-based assay (Promega) 24 hr after transfection and normalized to the β-galactosidase plasmid uptake as described in the Experimental Procedures. Fold activation in 293T cells is expressed relative to the basal expression of pHIV-LTR-Luc set as 1. Mean values of the luciferase activities represent at least three independent transfection experiments.

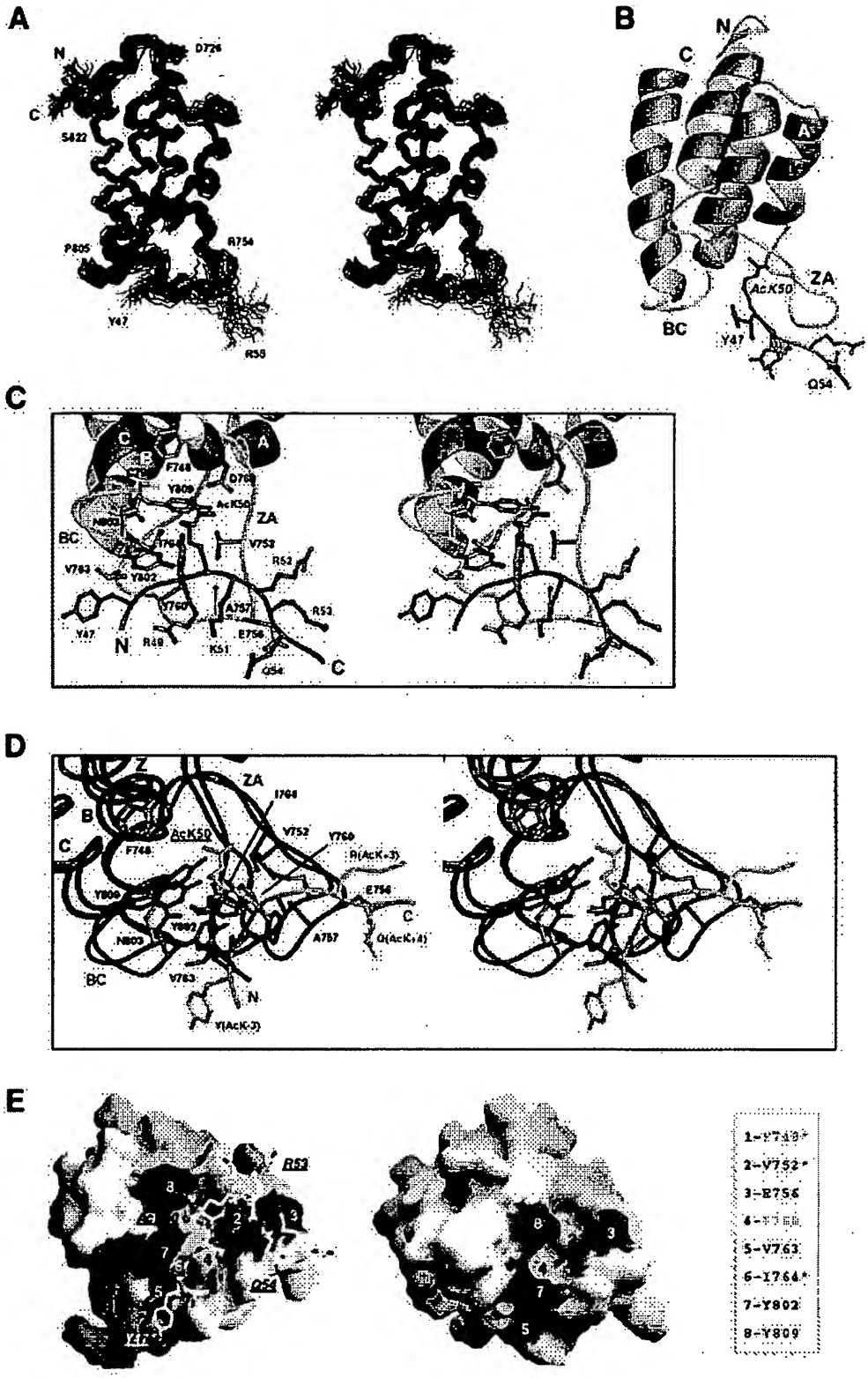


Figure 2. NMR Structure of the PCAF Bromodomain/Tat AcK50 Peptide Complex

(A) Stereoview of the backbone atoms (N, Cα, and C') of 25 superimposed NMR-derived structures of the PCAF bromodomain (black) (showing residues 719–830) in complex with the Tat AcK50 peptide (green) (showing residues 46–55). Note that amino acid residues in the Tat peptide are described either according to their relative positions with respect to the acetyl-lysine in the sequence or for clarity numbered by their specific positions in the protein sequence of Tat.

Table 1. Summary of NMR Structural Statistics

Total Experimental Restraints	2903	
Total NOE Distance Restraints[a]	2822	
Protein		
Total ambiguous	122	
Total unambiguous	2590	
Intraresidue	1093	
Interresidue		
Sequential ($\|i - j\| = 1$)	480	
Medium ($2 \leq \|i - j\| \leq 4$)	547	
Long range ($\|i - j\| > 4$)	470	
Peptide	32	
Intermolecular	78	
Hydrogen Bond Restraints	28	
Dihedral Angle Restraints	53	
Final Energies (kcal·mol^{-1})		
E_{Total}	366.4 ± 31.1	
E_{NOE}	58.0 ± 12.6	
$E_{Dihedral}$	0.6 ± 0.3	
E_{L-J}[b]	−569.5 ± 22.4	
	Protein/Peptide Complex[f]	Secondary Structure
Ramachandran Plot (%)		
Most favorable region	72.1 ± 2.3	92.0 ± 3.0
Additionally allowed region	22.9 ± 2.4	7.4 ± 3.1
Generously allowed region	3.6 ± 1.4	0.6 ± 0.1
Disallowed region	1.3 ± 0.6	0.0 ± 0.0
Cartesian coordinate RMSDs (Å)[c]		
Backbone atoms (N, Cα, and C′)[d]	0.66 ± 0.14	0.39 ± 0.05
Heavy atoms[d]	1.25 ± 0.18	0.96 ± 0.08
Backbone atoms (N, Cα, and C′)[e]	0.50 ± 0.16	
Heavy atoms[e]	1.83 ± 0.50	
Backbone atoms (N, Cα, and C′)[f]	0.72 ± 0.15	0.54 ± 0.09
Heavy atoms[f]	1.39 ± 0.20	1.25 ± 0.16

[a] Of the total 2822 NOE-derived distance restraints, 341 were obtained by using ARIA program, of which 122 are classified as ambiguous NOEs. The latter NOE signals in the NMR spectra match with more than one proton atom in both the chemical shift assignment and the final NMR structures.
[b] The Lennard-Jones potential was not used during any refinement stage.
[c] None of these final structures exhibit NOE-derived distance restraint violations greater than 0.3 Å or dihedral angle restraint violations greater than 5°.
[d] Protein residues 723–830.
[e] Peptide residues 47–52 and 53–54.
[f] Protein residues 723–830 and peptide residues 47–52 and 53–54.

residues (Figure 1B; see Supplemental Figure S1 at http://www.molecule.org/cgi/content/full/9/3/575/DC1). For instance, aromatic protons of Y802 in the BC loop and Y760 in the ZA loop show numerous long-range NOEs in the free form, which become completely absent in the peptide-bound form (see Supplemental Figures S2A, S2B, and S2C at http://www.molecule.org/cgi/content/full/9/3/575/DC1). These changes of NOE patterns are reflected in a ~90° rotational flip of the aromatic ring of Y802, which opens a channel lined by the ZA and BC loops to grasp the peptide through intermolecular interactions such as those observed between Y(AcK-3) and V763 (Figure 2D). Changes of loop conformation in the ZA and BC loops also result in exposing otherwise almost completely buried protein residues such as F748, V752, and I764 for direct peptide recognition (Figure 2E). Supporting NMR data include: (1) the methyl group (δ1) of I764 in the ZA loop shows a NOE cross peak to H^{α} of Y802, only in the free but not in the complex form; and (2) the methyl group of A757 in the ZA loop changes its spatial position from being close to the aromatic ϵ protons of Y802 (Y802.ϵ) in the free form to being proximal to Y761.ϵ upon binding to the Tat peptide (see Supplemental Figures S2D and S2E at

(B) Ribbons (Carson, 1991) representation of the average minimized NMR structure of the PCAF bromodomain/Tat peptide complex.
(C) Stereoview of the Tat binding site in the bromodomain showing side chains of the protein (green) and peptide (blue) residues that are directly involved intermolecular interactions.
(D) Stereoview of superimposition of the free (green) and ligand-bound (blue) structures of PCAF bromodomain showing side chain conformation of the residues in the Tat peptide binding site. The residues of the Tat peptide are colored in orange.
(E) Surface representation of the Tat binding site of the bromodomain in ligand-bound (left) and free form (right). Protein residues important in ligand recognition are colored with the same color scheme in both structures. Residues indicated by an asterisk are almost completely buried in the free form structure.

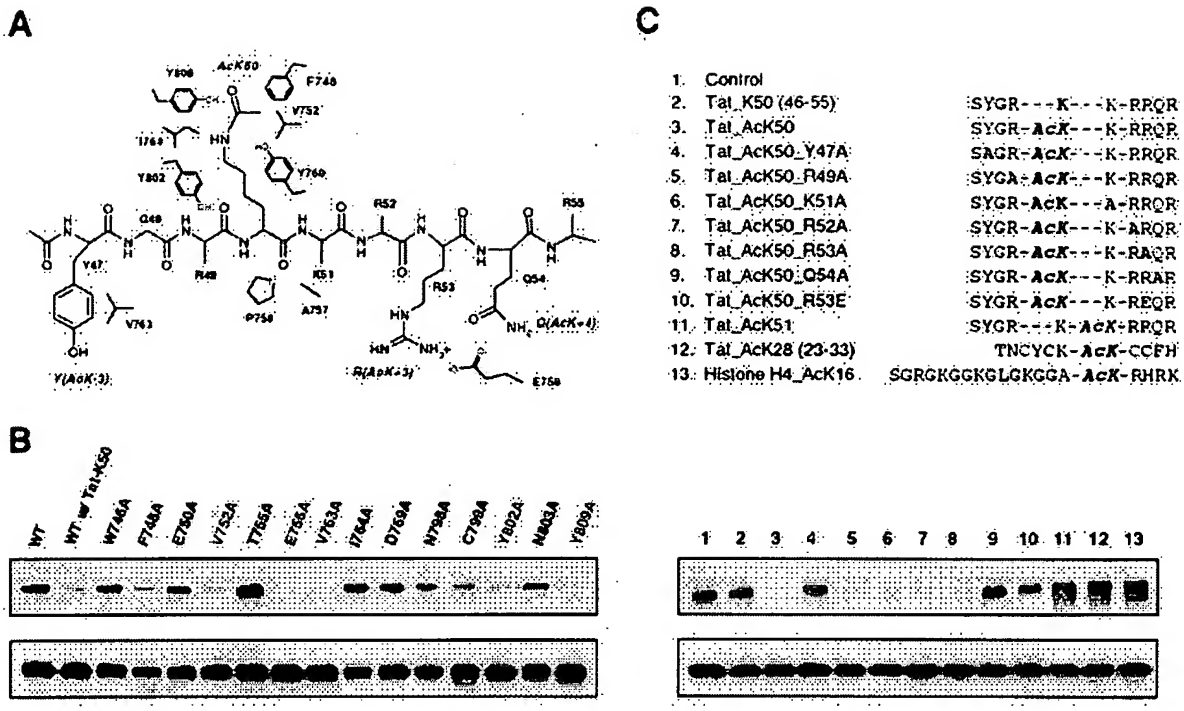


Figure 3. Mutational Analyses of PCAF Bromodomain Binding to HIV-1 Tat

(A) Schematic diagram showing amino acid residues involved in the protein/peptide interface.

(B) Effect of point mutation of protein residues on Tat AcK50 peptide binding. Western blot with anti-GST antibodies shows binding of the GST-fusion PCAF bromodomain proteins to the biotinylated Tat AcK50 peptide immobilized on streptavidin agarose beads (upper panel). The lower panel indicates a relatively equal amount of bromodomain proteins used in each binding experiment. Protein residues highlighted in red exhibited a significant reduction in binding to the Tat AcK50 peptide due to an alanine substitution.

(C) Mutational analysis of Tat peptide residues. Mutation effect was assessed by a peptide competition assay using anti-GST Western blot, in which a nonbiotinylated peptide competes with the biotinylated wild-type Tat AcK50 peptide for binding to the GST-fusion PCAF bromodomain. Numerals above the upper panel indicate a specific peptide used in the competition assay. The numerals in red refer to mutant Tat peptides that showed a significant reduction in binding to the bromodomain in the competition assay. The lower panel shows the relatively equal amount of bromodomain proteins used in each study. For clarity, the peptide residues are numbered according to their positions in the Tat protein sequence.

http://www.molecule.org/cgi/content/full/9/3/575/DC1). Together, our NMR data strongly suggest that conformational changes of protein residues in the ligand binding site are directly coupled with the highly selective interactions between PCAF bromodomain and acetylated Tat.

Specificity of PCAF Bromodomain and Tat Recognition

To determine the relative contributions of bromodomain residues in Tat binding site (Figure 3A), we examined its mutant proteins for binding to an N-terminal biotinylated Tat AcK50 peptide immobilized onto streptavidin agarose beads. Mutation of bromodomain residues W746, E750, T755, I764, D769, N798, C799, or N803 to alanine did not affect peptide binding, whereas proteins containing an alanine mutation at F748, V752, Y802, or Y809 showed a major reduction or nearly complete loss in Tat binding (Figure 3B). Moreover, alanine substitution of V763 or E756 almost completely abrogated peptide association, underlining the importance of their specific interactions with peptide residues Y(AcK−3) and Q(AcK+4). It is important to note that since both V763 and E756 are solvent exposed and located in loop regions of the structure (Figure 2D), their individual mutation to alanine unlikely causes major conformational disruption of the protein. Mutation results of these protein residues are consistent with the NMR structure of the complex and confirm their direct interactions with the acetyl-lysine and/or its flanking residues in the Tat peptide.

To further identify determinants in Tat sequence for PCAF bromodomain recognition, we synthesized mutant peptides and tested their binding to the protein in a peptide competition assay. Because of the high sensitivity of this detection method, the binding study was performed at a protein concentration (10 μM) much lower than that required for NMR study (~200 μM), ensuring specificity of protein-peptide interactions. As anticipated, lysine-acetylated peptides derived from Tat at K51 or K28 (lanes 11 and 12 in Figure 3C) or from histone H4 at K16 (lane 13) showed almost no competition against Tat AcK50 peptide in PCAF bromodomain binding, demonstrating that the latter interaction is of high affinity and specificity. Alanine substitution of resi-

dues R(AcK−1), K(AcK+1), R(AcK+2), or R(AcK+3) in Tat AcK50 peptide slightly weakened its binding to the bromodomain. Conversely, change of Y(AcK−3) (lane 4) or Q(AcK+4) (lane 9) to alanine caused a nearly complete loss of bromodomain binding, confirming the importance of their pairwise interactions with V763 and E756 for Tat-PCAF association. Finally, while mutation of R(AcK+3) to alanine (lane 8) did not significantly alter Tat binding to the bromodomain, its substitution to glutamic acid (lane 10) exhibited a marked reduction in the protein/peptide interaction. The effect of the latter mutation is likely due to an electrostatic repulsion between the glutamate and E756 of the protein. Together, these results explain the structural basis for the highly selective nature of PCAF and lysine-acetylated Tat association, which requires specific interactions of the bromodomain with AcK50 and its flanking residues, including Y(AcK−3), R(AcK+3), and Q(AcK+4).

PCAF Bromodomain Competing against TAR RNA for Binding to Lysine-Acetylated Tat

The arginine-rich motif containing R52 and R53 in Tat is also known to interact with the HIV-1 TAR RNA element (Aboul-ela et al., 1995; Long and Crothers, 1999; Rana and Jeang, 1999). Tat acetylation at K50 by p300/CBP promotes Tat dissociation from TAR RNA in early transcriptional elongation (Deng et al., 2000; Kiernan et al., 1999). To determine whether lysine acetylation directly affects Tat association with TAR RNA, we performed an NMR study of a 27 nucleotide HIV-1 TAR RNA binding to Tat peptides containing either K50 or AcK50. Our results showed that TAR RNA bound to the nonacetylated Tat peptide with a subnanomolar affinity (K_D), in agreement with results reported previously (Aboul-ela et al., 1995; Long and Crothers, 1999), and that K50 acetylation of Tat resulted in a significant reduction of its affinity to TAR RNA (data not shown). More strikingly, we found that PCAF bromodomain competes effectively against TAR RNA for binding to Tat AcK50 peptide (Figures 4A and 4B), suggesting that the binding affinity (K_D) of the latter interaction is on the order of low micromolar. This observation may be explained by possible conformational change of the peptide residues due to acetylation at K50 or involvement of R53 of Tat in both interactions. These results strongly imply that the PCAF bromodomain interaction with AcK50 on Tat not only contributes to Tat-PCAF association but also to the release of lysine-acetylated Tat from TAR RNA association, leading to Tat-mediated HIV-1 transcriptional activation.

Differences of Ligand Selectivity of Bromodomains

Structural comparison of bromodomains from PCAF and other proteins extends our understanding of differences in ligand selectivity. Recent structures of bromodomains from human GCN5 (Hudson et al., 2000) and *Saccharomyces cerevisiae* GCN5p (Owen et al., 2000), and the double bromodomain module of human $TAF_{II}250$ (Jacobson et al., 2000), reinforce the notion that the left-handed four-helix bundle fold of the PCAF bromdomain is conserved in the bromodomain family (Dhalluin et al., 1999). Structural similarity is high for the four helices with pairwise root-mean-square deviations of 0.7–1.8 Å for the backbone Cα atoms. The majority of structural deviations are localized in the loop regions, particularly in the ZA and BC loops (see Supplemental Figure S3 at http://www.molecule.org/cgi/content/full/9/3/575/DC1).

The crystal structure of scGCN5p bromodomain solved in complex with an acetylated peptide derived from histone H4 at K16 (A-AcK-RHRKILRNSIQGI) reveals that the mechanism of acetyl-lysine recognition is highly conserved in bromodomains—it involves a nearly identical set of corresponding conserved residues in the PCAF and scGCN5p bromodomains (Figures 5A and 5B) (Owen et al., 2000). In addition to the acetyl-lysine, scGCN5p bromodomain has a limited number of contacts with two residues at (AcK+2) and (AcK+3) in the H4 peptide. Binding of H(AcK+2) to aromatic rings of Y406 and F367 in scGCN5p is reminiscent of PCAF bromodomain recognition of Tat Y(AcK−3) through interactions with Y802 and V763, which are equivalent to the two scGCN5p residues. Because of this similar mode of molecular interaction, the two aromatic residues in the Tat and H4 peptides, which are located in very different positions with respect to the acetyl-lysine, are bound in a nearly identical position in the corresponding bromodomain structures (Figure 5A). High conservation of these residues in bromodomains (Figure 5B) suggests that selection of an aromatic or hydrophobic residue neighboring the acetyl-lysine is possibly conserved for many members of the bromodomain family.

It is important to note that while the major binding determinant in scGCN5p bromodomain-H4 complex is the acetyl-lysine (Owen et al., 2000), the highly specific association of PCAF bromodomain and Tat peptide is dependent on its interactions not only with the acetyl-lysine and Y(AcK−3) but also with residues on the other side of the acetyl-lysine at (AcK+3) and (AcK+4) (Figures 3B and 3C). These differences in the extent of ligand interactions explain why the Tat AcK50 peptide competes effectively against a similar histone H4 AcK16 peptide for binding to the PCAF bromodomain (Figure 3C, lane 13). Moreover, these differences in ligand selectivity provide an explanation for the striking differences in location and orientation of the bound peptides in the two bromodomains—the backbones of the Tat and H4 peptides lie in the two corresponding structures nearly antiparallel to each other (Figure 5A). Binding of A757 and E756 in the ZA loop to R(AcK+3) and Q(AcK+4) of the Tat peptide, which are completely lacking in the scGCN5 bromodomain-H4 complex, further explains why the PCAF bromodomain undergoes more extensive conformational changes in the ligand site than those seen in the GCN5 bromodomains (see Supplemental Figure S3 at http://www.molecule.org/cgi/content/full/9/3/575/DC1). While the biological relevance of the scGCN5 and histone H4 AcK16 interaction remains to be determined, a growing body of evidence, including previous reports (Benkirane et al., 1998; Deng et al., 2000), our present study of NMR structure and in vitro mutagenesis, and results from in vivo functional studies of Tat-mediated HIV-1 transcriptional activation (Figure 1C and M.O. and E.V., unpublished data), strongly support the biological relevance and importance for the highly selective association of PCAF bromodomain and acetylated Tat.

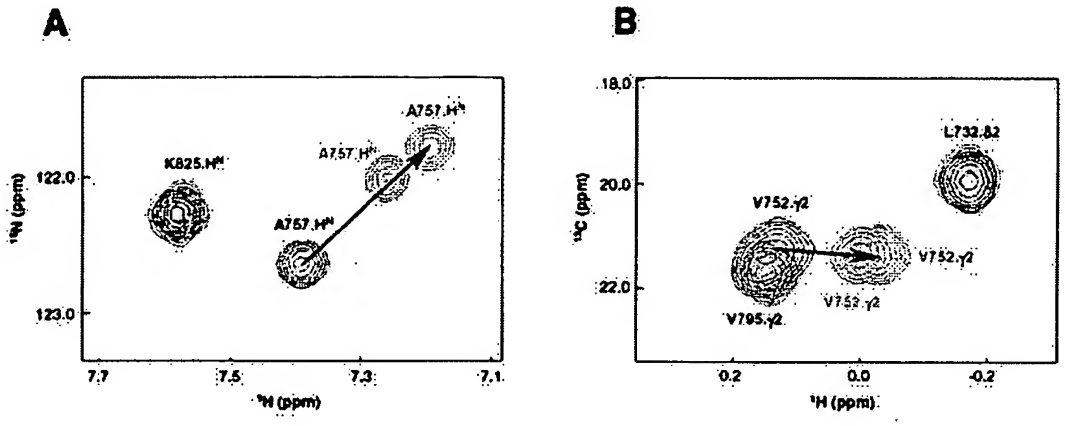


Figure 4. PCAF Bromodomain Competing against TAR RNA for Binding to Tat AcK50 Peptide

(A) Superimposition of a selected region of 2D ^{1}H-^{15}N HSQC spectra of a $^{13}C/^{15}N$-labeled PCAF bromodomain protein in the free form (black), in the presence of Tat AcK50 peptide (molar ratio of 1:1.2) (red), and in the presence of Tat AcK50 peptide and TAR RNA (molar ratio of 1:1.2:1 for protein:peptide:RNA) (blue). The spectra show chemical shift changes of the backbone amide resonances of protein residues due to peptide binding.

(B) Superimposition of 2D ^{1}H-^{13}C HSQC spectra of the PCAF bromodomain collected under the same conditions as described in (A). The NMR spectra exhibit chemical shift changes of the side chain methyl group resonances of protein residues due to peptide binding. The same color-coding scheme was used as in (A). Arrows indicate chemical shift changes of protein NMR resonances from the free form (black) to the Tat AcK50 peptide-bound form (red). Note that only the bromodomain residues (i.e., A757 and V752) that directly interact with the Tat peptide, as shown in the three-dimensional structure, exhibited major chemical shift changes upon peptide binding or in competing against TAR RNA for binding to the Tat peptide. More importantly, addition of TAR RNA causes only small shifts of the protein signals from the Tat peptide-bound position toward the free form position, suggesting that the PCAF bromodomain competes effectively against TAR RNA for binding to the lysine-acetylated Tat peptide. We observed by NMR no significant nonspecific interactions between the protein and TAR RNA under these conditions.

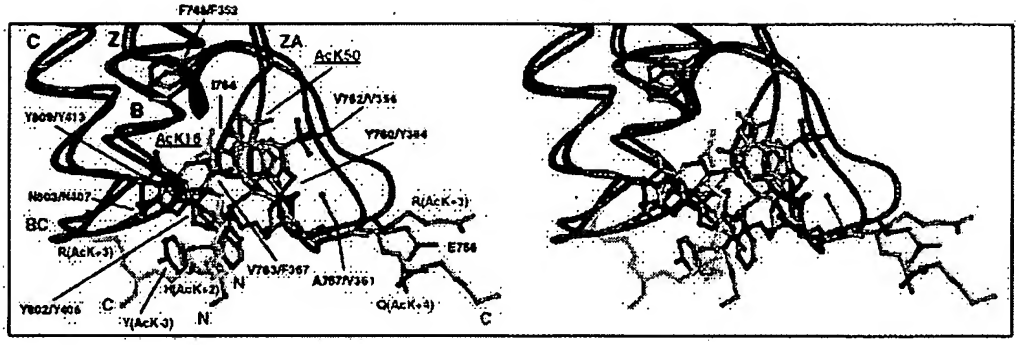
Since bromodomain residues important for acetyl-lysine recognition are largely conserved, binding of acetyl-lysine on a protein is likely a general biochemical function for bromodomains. However, differences in ligand selectivity may be attributed to a few but important differences in bromodomain sequences (Figure 5B), which include (1) variations in the ZA loop such as relatively low sequence conservation and amino acid deletion or insertion; and (2) variation of bromodomain residues that are involved in direct interactions with residues surrounding acetyl-lysine in a target protein. For instance, E756 in the bromodomain of PCAF is unique and only present in a small subset of bromodomains including GCN5. An analogous residue in the structurally similar bromodomain of CBP or p300 (Y.H. and M.-M.Z., unpublished data) is a leucine followed by a 2 amino acid insertion, which are present in a small subfamily of bromodomains (Figure 5B) (Jeanmougin et al., 1997). Moreover, a short helix corresponding to the AWPFM sequence in the ZA loop of PCAF (residues 745–749) is likely completely missing in TIF1β bromodomain due to amino acid deletion, and E756 of PCAF is substituted with a two residue AT motif in the sequence. Together, these findings explain why bromodomains from CBP and TIF1β did not interact with Tat AcK50 peptide (Figure 1A).

Human GCN5-S, a shorter version of hGCN5 that contains only the HAT domain and the bromodomain but lacks the N-terminal p300/CBP binding domain due to an alternative RNA splicing (Schiltz and Nakatani, 2000; Yamauchi et al., 2000), has been recently reported to interact with HIV Tat in vitro (Col et al., 2001). This Tat interaction involves both the HAT domain and the bromodomain of hGCN5-S. While the specific binding site on Tat by the hGCN5 bromodomain and the question of whether this bromodomain interaction is dependent on Tat lysine acetylation remain to be determined, our data reported here suggest that the hGCN5 bromodomain may possess ligand selectivity similar to that of the structurally homologous bromodomain of PCAF.

Conclusion

Tat-stimulated transcriptional activation of integrated HIV-1 genomes defines the rate-limiting step for viral replication (Adams et al., 1994; Benkirane et al., 1998). Tat synergy with histone acetyltransferases and its recruitment of PCAF via a bromodomain interaction, as we described in this study, support the notion that Tat transactivation of HIV-1 chromosomal transcription proceeds via chromatin remodeling (Deng et al., 2000; Kiernan et al., 1999; Ott et al., 1999). Our findings may explain why deletion of the PCAF C-terminal region comprising the bromodomain potently abrogated Tat transactivation of integrated but not unintegrated HIV-1 LTR (Benkirane et al., 1998). Our study reinforces the concept that bromodomain and acetyl-lysine recognition could serve as a pivotal mechanism for controlling protein-protein interactions in chromatin remodeling as well as other cellular processes including viral life cycle (Dyson et al., 2001; Winston and Allis, 1999), and that differences

A



B

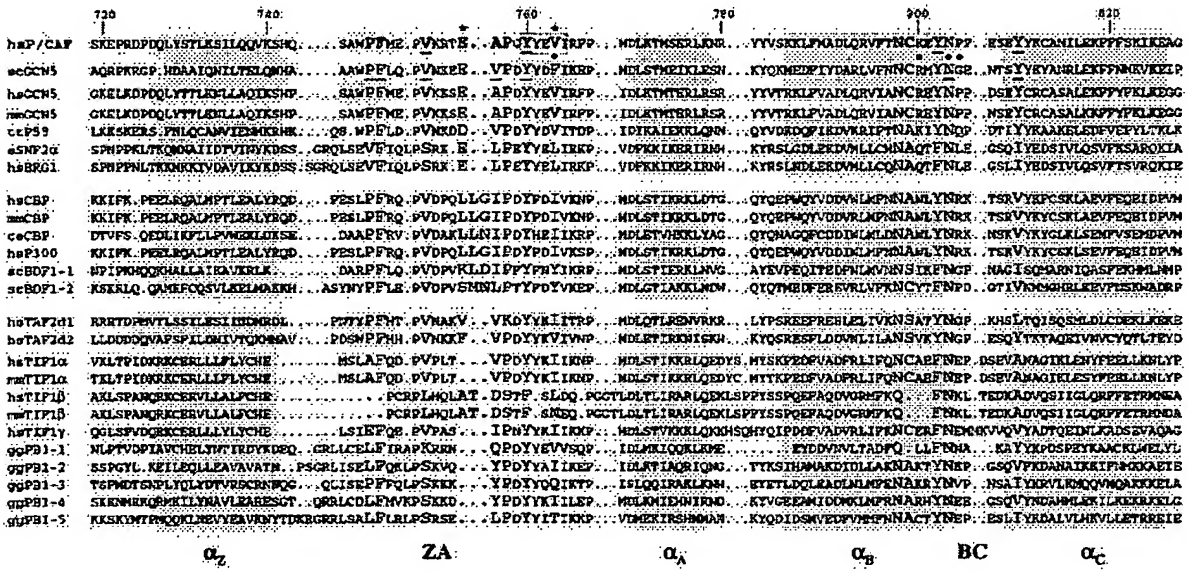


Figure 5. Differences in Ligand Selectivity of Bromodomains

(A) Stereoview of superimposition of the structures of the PCAF bromodomain-Tat peptide complex (blue) and the scGCN5 bromodomain-H4 peptide complex (red) showing conformational differences of protein residues in the peptide binding sites. The lysine-acetylated peptides of HIV-1 Tat and histone H4 are shown in green and orange, respectively. The residues of the PCAF and scGCN5 bromodomains are numbered according to protein sequences and color-coded in black and red, respectively. The corresponding conserved residues in the two bromodomains are annotated together. The amino acid residues of the Tat and H4 peptides are described according to their position with respect to the acetyl-lysine in the corresponding peptide sequences. The structures were superimposed on the four helices of the two bromodomains in Insight and the figure was prepared with Ribbons.

(B) Sequence alignment of a selected number of bromodomains. The sequences were aligned based on the experimentally determined three-dimensional structures of five bromodomains, highlighted in yellow. Note that in the PCAF bromodomain, a short helix in the ZA loop comprising the YYEVI sequence (residues 760–764) (boxed by dashed lines) was only observed in the free form but not in the Tat peptide-bound form. The predicted secondary structures in the corresponding regions of other bromodomains are shown in green. Because of relatively high variations in amino acid sequence in the ZA loop, prediction of secondary structures was omitted. Bromodomains are grouped on the basis of the predicted sequence and/or structural similarities. Residue numbers of the PCAF bromodomain are indicated above its sequence. Three absolutely conserved residues, corresponding to P751, P767, and N803 in the PCAF bromodomain, are shown in purple. Highly conserved residues are colored in blue. The residues in the PCAF bromodomain that directly interact with the Tat peptide, as determined by intermolecular NOEs, are displayed in a larger font size. The residues essential for the acetyl-lysine binding are underlined, and the residues important for ligand selectivity through interactions with the peptide residues flanking the acetyl-lysine are highlighted by red asterisks. The protein residues contacting the acetyl-lysine in the scGCN5 bromodomain-H4 peptide complex are underlined, and residues contacting other parts of the peptide are indicated by red dots.

in ligand selectivity of conserved protein modular domains can be achieved by evolutionary changes of amino acid sequences in the ligand binding site. The new knowledge of the structural basis of PCAF bromodomain and Tat recognition should aid in the design of small molecules that can be used to block this specific

interaction in order to disrupt HIV-1 transcriptional activation and replication.

Experimental Procedures

Sample Preparation

The PCAF bromodomain (residues 719–832) was expressed in *Escherichia coli* BL21(DE3) cells using the pET14b vector (Novagen) (Dhalluin et al., 1999). Isotope-labeled bromodomain proteins were prepared from cells grown on a minimal medium containing $^{15}NH_4Cl$ with or without $^{13}C_6$-glucose in either H_2O or 75% ^2H_2O. The protein was purified by affinity chromatography on a nickel-IDA column (Invitrogen), followed by the removal of poly-His tag by thrombin cleavage. GST-fusion bromodomains from PCAF, CBP, and TIF1β were expressed in *E. coli* BL21 (DE3) codon plus cells using the pGEX4T-3 vector (Pharmacia) and purified with a glutathione sepharose column. NMR spectra of the recombinant CBP and TIF1β proteins were acquired to ensure that they were properly folded and functional (see Supplemental Figure S4 at http://www.molecule.org/cgi/content/full/9/3/575/DC1). The acetyl-lysine-containing peptides were prepared on a MilliGen 9050 peptide synthesizer (Perkin Elmer) using Fmoc/HBTU chemistry. Acetyl-lysine was incorporated using the reagent Fmoc-Ac-Lys with HBTU/DIPEA activation. The HIV-1 TAR RNA was obtained from Dharmacon Research, Inc. (Lafayette, CO).

NMR Spectroscopy

NMR samples contained a protein/peptide complex of 0.5 mM in 100 mM phosphate buffer (pH 6.5) containing 5 mM perdeuterated DTT and 0.5 mM EDTA in $H_2O/^2H_2O$ (9/1) or ^2H_2O. All NMR spectra were acquired at 30°C on a Bruker 500 or 600 MHz NMR spectrometer. The ^1H, ^{13}C, and ^{15}N resonances of the protein backbone and side chain atoms were assigned by using a standard set of triple-resonance experiments (Sattler et al., 1999) with a uniformly $^{13}C/^{15}N$-labeled and 75% deuterated protein in complex with an unlabeled peptide. The distance restraints were obtained from ^{13}C- or ^{15}N-edited three-dimensional nuclear Overhauser enhancement spectroscopy (NOESY) spectra (Clore and Gronenborn, 1994). ϕ-angle restraints were determined based on the $^3J_{HNH\alpha}$ coupling constants measured in a 3D HNHA spectrum (Clore and Gronenborn, 1994). Slowly exchanging amide protons were identified from a series of 2D ^{15}N-HSQC spectra recorded after the H_2O buffer was changed to a ^2H_2O buffer, which were used together with the initial structures calculated with only NOE-derived distance restraints to generate hydrogen-bond distance restraints in final structure calculations. The intermolecular NOEs were detected in ^{13}C-edited (F_1), $^{13}C/^{15}N$-filtered (F_3) 3D NOESY spectrum (Clore and Gronenborn, 1994). All NMR spectra were processed with the NMRPipe program (Delaglio et al., 1995) and analyzed using NMRView (Johnson and Blevins, 1994). NMR binding studies of Tat peptides and TAR RNA interactions were performed in the phosphate buffer (pH 6.5) containing 200 mM NaCl to minimize any nonspecific interactions.

Structure Calculations

Structures of the protein/peptide complex were calculated with a distance geometry-simulated annealing protocol using the X-PLOR program (Brunger, 1993). A total of 2359 manually assigned NOE-derived distance restraints were used in initial structure calculations. The ARIA (Nilges and O'Donoghue, 1998)-assigned distance restraints agree with the structures calculated using only the manually determined NOE distance restraints, 28 hydrogen-bond distance restraints, and 53 ϕ angle restraints. The final structure calculations employed a total of 2903 NMR experimental restraints from the manual and the ARIA-assisted assignments, including 2700 unambiguous intramolecular and 78 intermolecular NOE distance restraints. The distance restraint force constant was 50 kcal mol^{-1} Å^{-2}, and no NOE was violated by more than 0.3 Å. The torsion restraint force constant was 200 kcal mol^{-1} rad^{-2}, and no dihedral angle restraint was violated by more than 5°. While only the covalent geometry terms, NOE, torsion, and repulsive van der Waals terms were used in the structure refinement, a large, negative Lennard-Jones potential energy was observed (−569.5 ± 22.4 kcal mol^{-1}), indicating good nonbonded geometry of the structure. Procheck (Laskowski et al., 1996) analysis indicated that over 98% of the protein and peptide residues are in allowed regions of the Ramachandran map.

Mutational Analysis

Site-directed mutant proteins of PCAF bromodomain were prepared with the QuickChange kit (Stratagene). DNA sequencing confirmed the desired mutations. The GST-fusion bromodomains (10 μM) of PCAF, CBP, or TIF1β were incubated with an N-terminal biotinylated and lysine-acetylated Tat peptide (50 μM) in 50 mM Tris buffer (pH 7.5), containing 50 mM NaCl, 0.1% BSA, and 1 mM DTT at 22°C for 2 hr. Streptavidin agarose (10 μL) was added to the mixture, and the beads were washed in the Tris buffer containing 500 mM NaCl and 0.1% NP-40. Proteins eluted from the agarose beads were separated by SDS-PAGE and visualized by Western blotting using anti-GST antibody (Sigma) and horseradish-peroxidase-conjugated goat anti-rabbit IgG. Peptide competition assay was performed by incubating a nonbiotinylated peptide with the PCAF bromodomain and the biotinylated Tat AcK50 peptide. The molar ratio of the former and latter peptides in the mixture was kept at 1:2.

Plasmid Constructs

The mammalian expression vectors for the PCAF and CBP bromodomains were constructed as follows. Coding sequence for the PCAF bromodomain (residues 719–832) was subcloned into EcoRI-XhoI sites of pCMV-HA vector (Clontech). The CBP bromodomain (residues 1082–1197) was subcloned into BamHI-XhoI sites of pCMV-FLAG vector (Stratagene). The expression vectors for the full-length PCAF (pCI-FLAG-PCAF) (Li et al., 2000), the full-length CBP (pRSV-HA-CBP) (Kwok et al., 1996), HIV-1 Tat (pcTat), and the HIV-1 LTR-luciferase reporter construct (pHIV-LTR-Luc) (Bieniasz et al., 1998; Madore and Cullen, 1993) have been previously described.

Cell Culture and Transfections

Human 293T cells were propagated in Dulbecco's modified Eagle's medium with 10% fetal calf serum and transfected using the calcium phosphate coprecipitation method. Amounts of plasmid DNA used in cell transfections are as described in the legend to Figure 1C. The transfected 293T cells were lysed 24 hr after transfection and assayed for luciferase activity of the cell extracts using a luciferase-based assay system (Promega) (Bieniasz et al., 1998; Madore and Cullen, 1993). Luciferase activities derived from HIV-1 LTR were normalized to a cotransfected vector expressing β-galactosidase. The expression level of the transfected proteins was examined by Western blotting using monoclonal antibodies to HA (Roche Diagnostics), FLAG (Stratagene), or β-actin (Sigma), and rabbit polyclonal antibodies to the PCAF bromodomain or the CBP bromodomain (see Supplemental Figure S5 at http://www.molecule.org/cgi/content/full/9/3/575/DC1).

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Accession Numbers

Coordinates for the NMR three-dimensional structure of the PCAF bromodomain/HIV-1 Tat peptide complex have been deposited in the Brookhaven Protein Data Bank under the accession code 1JM4.

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(54) CONJUGATE VACCINE FOR NONTYPEABLE HAEMOPHILUS INFLUENZAE

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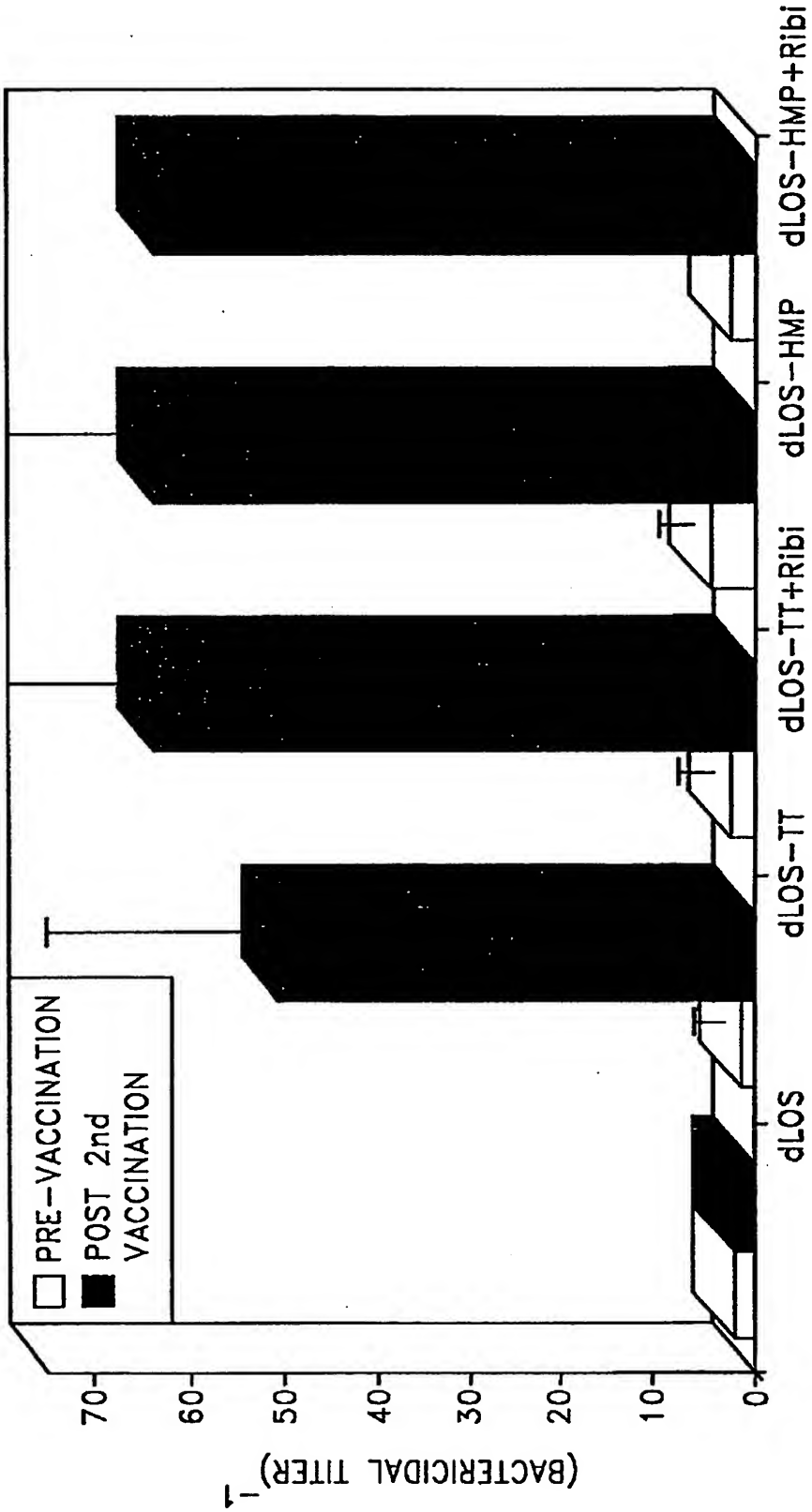
(57) ABSTRACT

A conjugate vaccine for Nontypeable Haemophilus influenzae comprising lipooligosaccharide from which esterified fatty acids have been removed conjugated to an immunogenic carrier. The vaccine is useful for prevention of otitis media and respiratory infections in mammals.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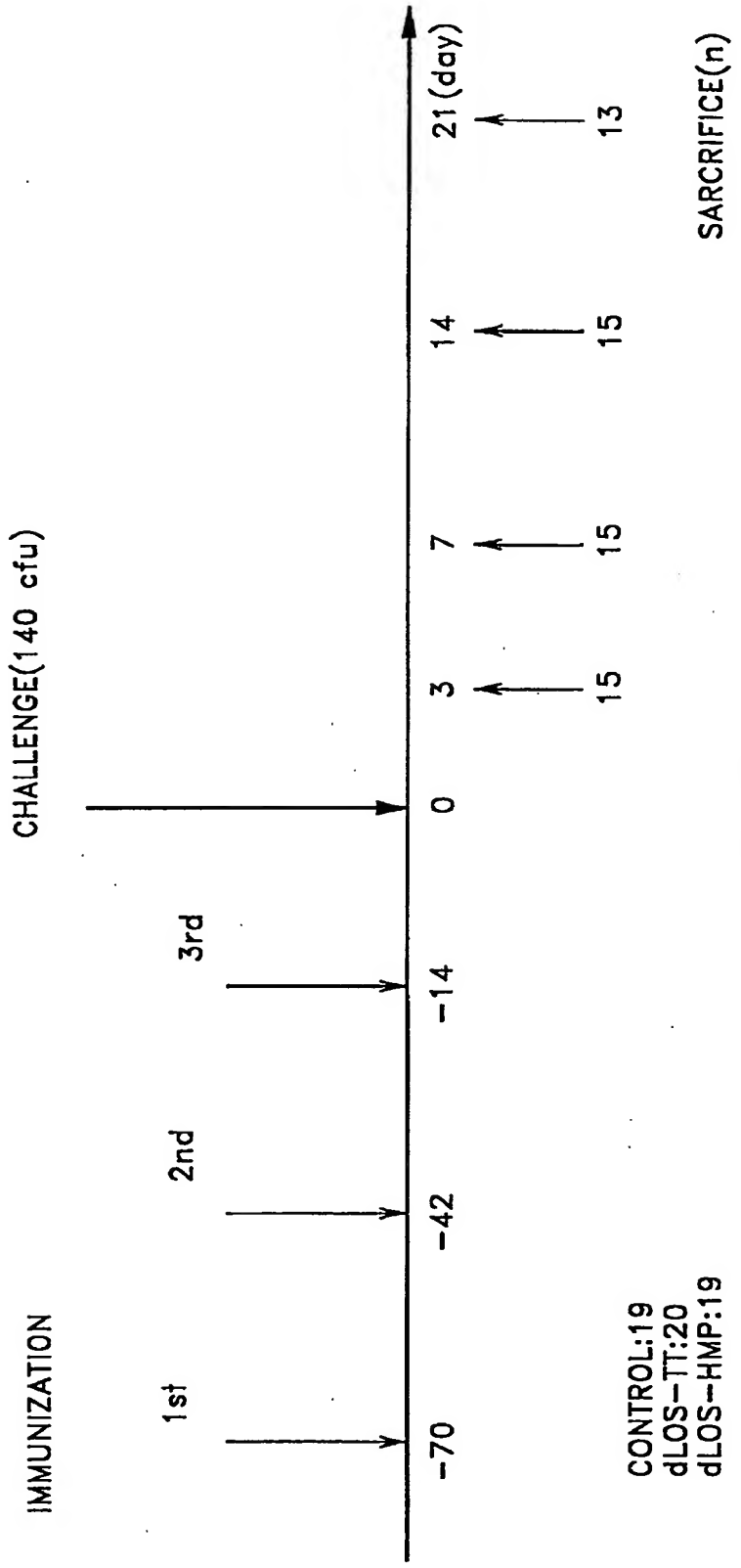
dLOS-TT+Ribi
dLOS-HMP
dLOS-HMP+Ribi

FIG. 1


IMMUNIZATION
1st
2nd
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0
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15
15
13
SARCRIFICE(n)
CONTROL:19
dLOS-TT:20
dLOS-HMP:19

FIG.2

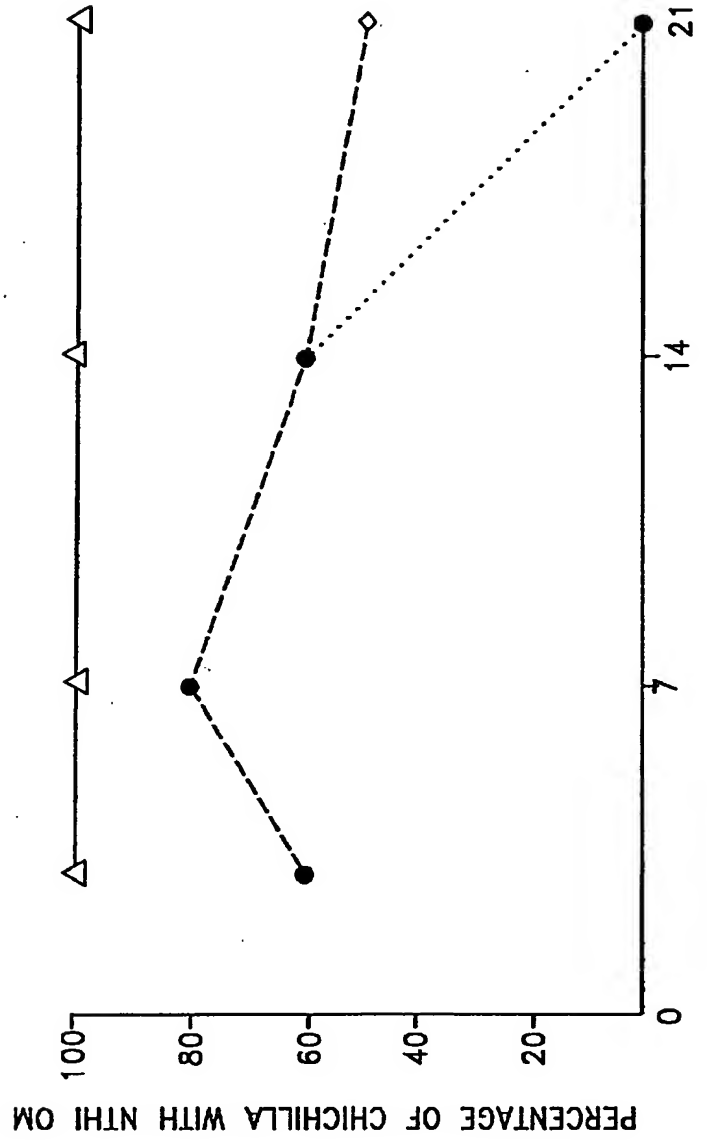
PERCENTAGE OF CHICHILLA WITH NTHI OM
100
80
60
40
20
0
7
14
21
DAYS POSTCHALLENGE
—△— CONTROL n=19
—○— dLOS-TT n=20
—◇— dLOS+HMP n=19

FIG. 3

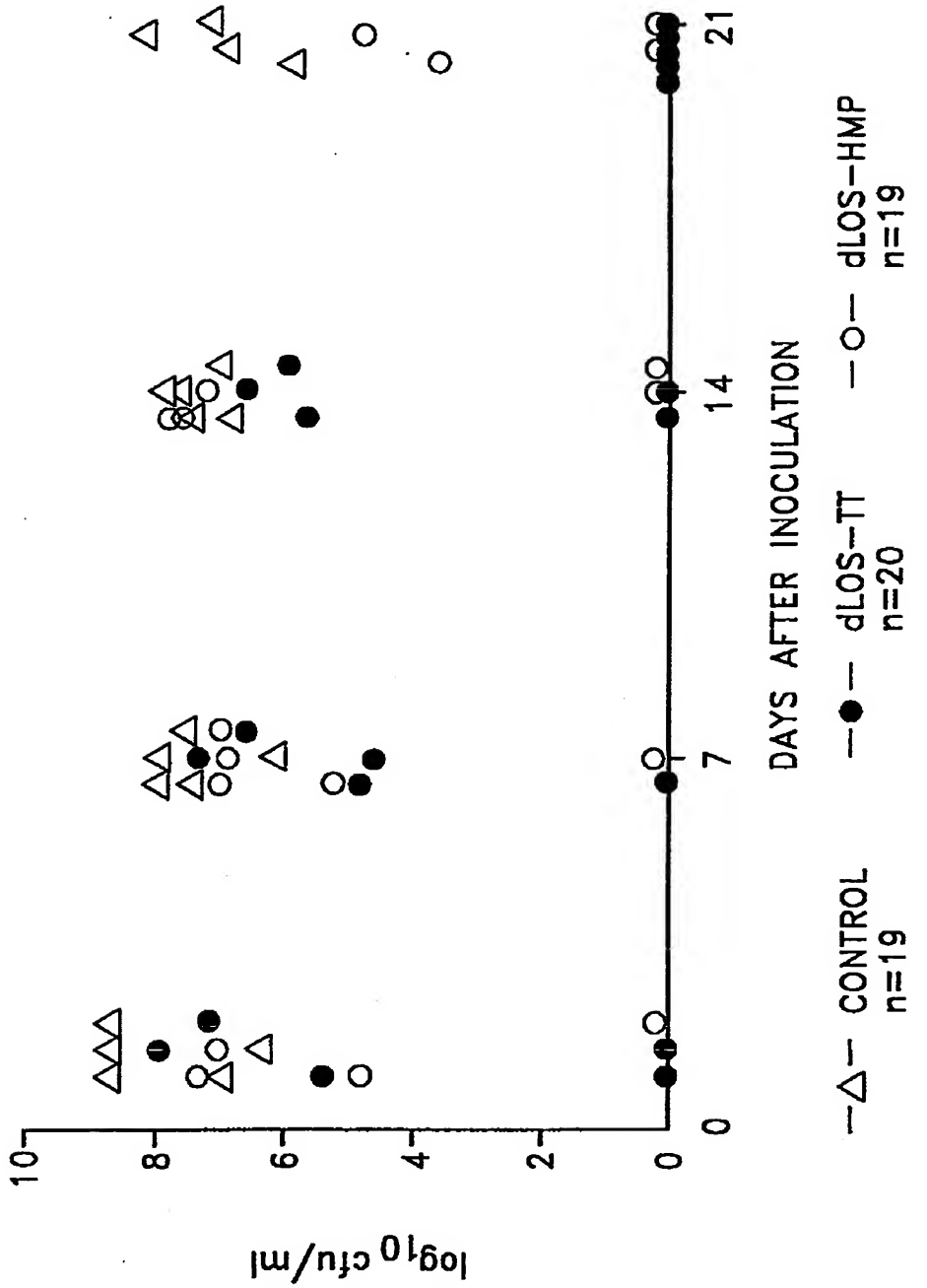
log10 cfu/ml
10
8
6
4
2
0
0
7
14
21
DAYS AFTER INOCULATION
—△— CONTROL n=19
—●— dLOS-TT n=20
—○— dLOS-HMP n=19

FIG. 4

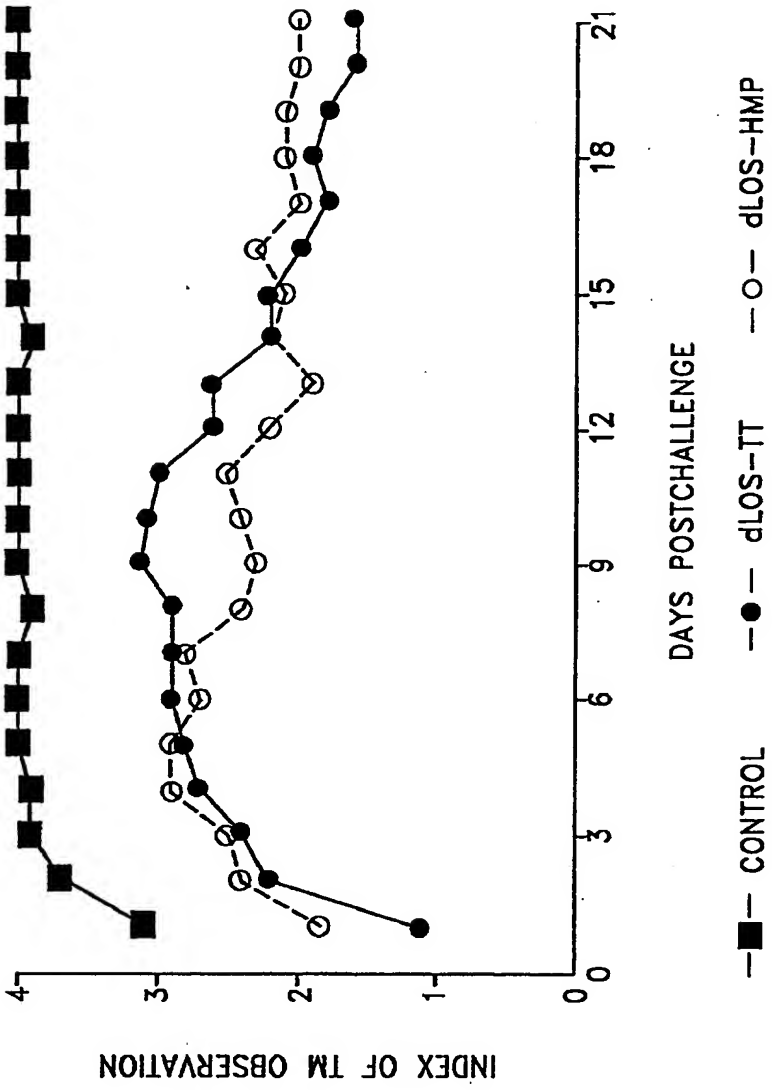
INDEX OF TM OBSERVATION
4
3
2
1
0
0
3
6
9
12
15
18
21
DAYS POSTCHALLENGE
CONTROL
dLOS-TT
dLOS-HMP

FIG.5

CONJUGATE VACCINE FOR NONTYPEABLE HAEMOPHILUS INFLUENZAE

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application claims priority under 35 U.S.C. § 1 19(e)(1) to U.S. Provisional Application Ser. No. 60/016,020, filed Apr. 23, 1996.

FIELD OF THE INVENTION

[0002] The present invention relates to conjugate vaccines for prevention of bacterial infections. More specifically, the invention relates to a conjugate vaccine for nontypeable Haemophilus influenzae comprising lipooligosaccharide from which esterified fatty acids have been removed linked to an immunogenic carrier.

BACKGROUND OF THE INVENTION

[0003] Nontypeable Haemophilus influenzae (NTHi) is a major causative agent for acute otitis media (middle ear infections) and respiratory infections. Acute otitis media and otitis media with effusion are common childhood diseases, second in frequency of occurrence only to the common cold (Stool et al., *Pediatr. Infect. Dis. Suppl.*, 8: S11-S14, 1989). The annual cost of the medical and surgical treatment of otitis media in the United States is estimated at between three and four billion dollars (Berman, *New Engl. J Med.*, 332:1560-1565, 1995). Moreover, inappropriate antibiotic treatment of otitis media can lead to the emergence of multidrug-resistant bacterial strains. There is currently no vaccine available for prevention of NTHi infection.

[0004] Current efforts in developing an NTHi vaccine are focused on cell surface antigens such as outer membrane proteins and pili or fimbria (Kyd et al., *Infect. Immun.*, 63:2931-2940, 1995; Deich et al., *Vaccine Res.*, 2:31-39, 1995). Among these, the most promising is P6 protein which appears to be antigenically conserved and elicits the production of antibodies that are bactericidal in vitro. Lipooligosaccharide (LOS) is a major NTHi cell surface antigen. LOS contains both lipid A and oligosaccharide (OS) components. Because the lipid A component of LOS is toxic, it must be detoxified prior to conjugation to an immunogenic carrier.

[0005] Barenkamp et al. (*Pediatr. Infect. Dis. J*, 9:333-339, 1990) demonstrated that LOS stimulated the production of bactericidal antibodies directed against NTHi. McGehee et al. (*Am. Journal Respir. Cell Biol.*, 1:201-210, 1989) showed that passive immunization of mice with monoclonal antibodies directed against LOS from NTHi enhanced the pulmonary clearance of NTHi.

[0006] Green et al. (*Vaccines*, 125-129, 1994) disclose an NTHi vaccine comprising a conjugate of NTHi OS and the mutant nontoxic diphtheria protein CRM197. The lipid A moiety was removed from LOS by treatment with acid, followed by derivatizing the resulting OS with adipic acid dihydrazide (ADH) and coupling to CRM_{197}. Despite the showing of Barenkamp et al. that LOS stimulated production of bactericidal antibodies against NTHi, the conjugates of Green et al. were determined to be poorly immunogenic after injection into mice. Moreover, the conjugates did not elicit bactericidal antibodies against NTHi.

[0007] Thus, there is a need for a vaccine effective against NTHi. The present invention satisfies this need.

SUMMARY OF THE INVENTION

[0008] One embodiment of the present invention is a conjugate vaccine for nontypeable Haemophilus influenzae (NTHi), comprising NTHi lipooligosaccharide from which esterified fatty acids have been removed (dLOS), and an immunogenic carrier covalently linked thereto. In another aspect of the present invention, the immunogenic carrier is a protein. Preferably, the protein is tetanus toxin/toxoid, NTHi high molecular weight protein, diphtheria toxin/toxoid, detoxified *P. aeruginosa* toxin A, cholera toxin/toxoid, pertussis toxin/toxoid, *Clostridium perfringens* exotoxins/toxoid, hepatitis B surface antigen, hepatitis B core antigen, rotavirus VP 7 protein, or respiratory syncytial virus F and G protein. Most preferably, the protein is tetanus toxoid or NTHi high molecular weight protein.

[0009] The present invention also provides a conjugate vaccine for nontypeable Haemophilus influenzae (NTHi), comprising NTHi lipooligosaccharide from which esterified fatty acids have been removed (dLOS), and an immunogenic carrier covalently linked thereto via a linker. Preferably, the linker is adipic acid dihydrazide, ϵ-aminohexanoic acid, chlorohexanol dimethyl acetal, D-glucuronolactone or p-nitrophenylamine; most preferably, the linker is adipic acid dihydrazide.

[0010] Another embodiment of the invention is isolated NTHi lipooligosaccharide detoxified by removal of ester-linked fatty acids therefrom.

[0011] The present invention also provides a pharmaceutical composition comprising the vaccine conjugates described above in a pharmaceutically acceptable carrier. The pharmaceutical composition may further comprise an adjuvant. Preferably, the adjuvant is alum.

[0012] Another embodiment of the invention is a method of preventing otitis media caused by NTHi in a mammal, comprising administering to the mammal an effective immunoprotective amount of the vaccine described above. Preferably, the mammal is a human. The route of administration may be intramuscular, subcutaneous, intraperitoneal, intraarterial, intravenous or intranasal; most preferably, the administering step is intramuscular. According to another aspect of this preferred embodiment, the effective dose is between about 10 μg and about 50 μg. The method may further comprise injecting between about 10 μg and about 25 μg at about 2 months and at about 13 months after the administering step. Alternatively, the method may further comprise injecting between about 10 μg and about 25 μg at about 2, 4 and 16 months after the administering step.

[0013] According to another aspect of the invention, there is provided a method of detoxifying lipooligosaccharide from NTHi, comprising removing ester-linked fatty acids therefrom. Preferably, the ester-linked fatty acids are removed by treating the LOS with hydrazine.

[0014] Still another aspect of the present invention is a method of making a conjugate vaccine against NTHi, comprising:

[0015] removing ester-linked fatty acids from NTHi lipooligosaccharide to produce DLOS; and

[0016] covalently binding said DLOS to an immunogenic carrier.

[0017] Advantageously, the removing step comprises treatment with hydrazine. The method may further comprise the step of attaching DLOS to a linker and attaching the linker to the carrier. Preferably, the linker is adipic acid dihydrazide, ε-aminohexanoic acid, chlorohexanol dimnethyl acetal, D-glucuronolactone or p-nitrophenylethyl amine; most preferably, the linker is adipic acid dihydrazide.

BRIEF DESCRIPTION OF THE DRAWINGS

[0018] **FIG. 1** shows the bactericidal activity of rabbit immune serum generated against dLOS, DLOS-TT, dLOS-TT plus adjuvant, dLOS-HMP and dLOS-HMP plus adjuvant. The y-axis shows the reciprocal of bactericidal titer. The reciprocal of bactericidal titer is proportional to bactericidal activity.

[0019] **FIG. 2** shows the timetable for administration of the DLOS-TT and dLOS-HMP conjugate vaccines and subsequent NTHi bacterial challenge in the chinchilla model of acute otitis media.

[0020] **FIG. 3** is a graph showing the percentage of chinchillas with NTHi otitis media up to 21 days postchallenge with NTHi.

[0021] **FIG. 4** is a graph showing bacterial counts in middle ear fluids from control and conjugate-immunized chinchillas at 3, 7, 14 and 21 days postchallenge with NTHi.

[0022] **FIG. 5** is a graph showing the index of tympanic membrane observation (TM) in control and conjugate-immunized chinchillas up to 21 days postchallenge with NTHi. The higher the index of TM observation, the more severe the otitis media.

DETAILED DESCRIPTION OF THE INVENTION

[0023] A conjugate vaccine comprising NTHi LOS from which esterified fatty acids had been removed (detoxified LOS or dLOS) and either tetanus toxoid (TT) or high molecular weight outer membrane adhesion proteins (HMPs) from NTHi induced significant increases in anti-LOS IgG antibody levels in both mice and rabbits. The isolation of HMPs is described by Barenkamp et al. (*Infect. Immun.* 64:1246-1251, 1996), the entire contents of which are hereby incorporated by reference. The HMPs exemplified herein are a mixture of two polypeptide species having approximate molecular weights of 120 kDa and 125 kDa. Additional HMPs having molecular weights of about 100 kDa are also described by Barenkamp et al. and are also contemplated for use in the present invention. Antigenicity of the two conjugates was similar to that of LOS. The antisera generated against the DLOS-TT and DLOS-HMP conjugates in rabbits was bactericidal against NTHi as determined by a complement-mediated cell lysis assay. The conjugates exhibited very low toxicity and were not pyrogenic in rabbits.

[0024] The DLOS-TT conjugate was studied in the chinchilla model of otitis media which is the art-recognized animal model for human otitis media. The dLOS-TT conjugate vaccine successfully protected chinchillas from a challenge dose of the NTHi strain used to make the vaccine (strain 9274). Chinchillas received injections of dLOS-TT conjugate from NTHi 9274 and were then challenged by intrabullar inoculation of NTHi 9274. Infection developed in all of the control animals and 56% of the conjugate-immunized animals over a time period of three weeks. The incidence of both left ear and inner ear infection was reduced 51% in the conjugate group relative to the control group. Among infected animals, bacterial counts of the middle ear fluids were significantly lower in conjugate-immunized animals than in control animals. All immunized animals responded with elevated serum titers of anti-LOS antibody and 49% demonstrated complement-mediated bactericidal activity against the homologous strain. Thus, active immunization with conjugates results in a significant in vivo reduction of the incidence of NTHi-induced otitis media in an art-recognized animal model of human otitis media.

[0025] Although the use of hydrazine for detoxification of LOS from NTHi is described herein, the use of any reagent or enzyme capable of removing ester-linked fatty acids from lipid A is within the scope of the present invention. Dried LOS from any strain of NTHi is suspended in liquid anhydrous hydrazine at a temperature of between 1° C. and 100° C.; preferably between 25° C. and 75° C.; more preferably, about 37° C. for a period between 1 hour and 24 hours, most preferably for a period of about 2-3 hours. After removal of ester-linked fatty acids, DLOS is conjugated to the linker adipic acid dihydrazide (ADH) prior to conjugation to the immunogenic carrier proteins TT or NTHi HMPs. Although ADH is the preferred linker, the use of any linker capable of stably and efficiently conjugating dLOS to an immunogenic carrier protein is contemplated. The use of linkers is well known in the conjugate vaccine field (see Dick et al., *Conjugate Vaccines*, J. M. Cruse and R. E. Lewis, Jr., eds., Karger, New York, pp. 48-114, the entire contents of which are hereby incorporated by reference). -DLOS may be directly covalently bonded to the carrier. This may be accomplished, for example, by using the cross linking reagent glutaraldehyde. However, in a preferred embodiment, DLOS and the carrier are separated by a linker. Presence of a linker promotes optimum immunogenicity of the conjugate and more efficient coupling of the dLOS with the carrier. Linkers separate the two antigenic components by chains whose length and flexibility can be adjusted as desired. Between the bifunctional sites, the chains can contain a variety of structural features, including heteroatoms and cleavage sites. Linkers also permit corresponding increases in translational and rotational characteristics of the antigens, increasing access of the binding sites to soluble antibodies. Besides ADH, suitable linkers include, for example, heterodifunctional linkers such as ε-aminohexanoic acid, chlorohexanol dimethyl acetal, D-glucuronolactone and p-nitrophenyl amine. Coupling reagents contemplated for use in the present invention include hydroxysuccinimides and carbodiimides. Many other linkers and coupling reagents known to those of ordinary skill in the art are also suitable for use in the invention. Such compounds are discussed in detail by Dick et al., supra.

[0026] The presence of a carrier increases the immunogenicity of the polysaccharide. In addition, antibodies raised against the carrier are medically beneficial. Polymeric immunogenic carriers can be a natural or synthetic material containing a primary and/or secondary amino group, an azido group or a carboxyl group. The carrier may be water soluble or insoluble.

[0027] Any one of a variety of immunogenic carrier proteins may be used in the conjugate vaccine of the present invention. Such classes of proteins include pili, outer membrane proteins and excreted toxins of pathogenic bacteria, nontoxic or "toxoid" forms of such excreted toxins, nontoxic proteins antigenically similar to bacterial toxins (cross-reacting materials or CRMs) and other proteins. Nonlimiting examples of bacterial toxoids contemplated for use in the present invention include tetanus toxin/toxoid, diphtheria toxin/toxoid, detoxified *P. aeruginosa* toxin A, cholera toxin/toxoid, pertussis toxin/toxoid and *Clostridium perfringens* exotoxins/toxoid. The toxoid forms of these bacterial toxins is preferred. The use of viral proteins (i.e. hepatitis B surface/core antigens; rotavirus VP 7 protein and respiratory syncytial virus F and G proteins) is also contemplated.

[0028] CRMs include CRM_{197}, antigenically equivalent to diphtheria toxin (Pappenheimer et al., *Immunochem.*, 9:891-906, 1972) and CRM3201, a genetically manipulated variant of pertussis toxin (Black et al., *Science*, 240:656-659, 1988). The use of immunogenic carrier proteins from non-mammalian sources including keyhole limpet hemocyanin, horseshoe crab hemocyanin and plant edestin is also within the scope of the invention.

[0029] There are many coupling methods which can be envisioned for dLOS-protein conjugates. In the examples set forth below, dLOS is selectively activated by 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC)-mediated ADH derivatization of the terminal 3-deoxy-D-manno-2-octulosonic acid (KDO) group of dLOS, followed by EDC-mediated coupling to TT. Alternatively, another method for producing the instant conjugates involves cystanine derivatization of DLOS, by, for example, EDC-mediated derivatization, followed by disulfide conjugation to N-succimidyl-3-(2-pyridyldithio) propionate-derivatized protein. Other methods well known in the art for effecting conjugation of oligosaccharides to immunogenic carrier proteins are also within the scope of the invention. Such methods are described in, for example, U.S. Pat. Nos. 5,153,312 and 5,204,098; EP 0 497 525; and EP 0 245 045, the entire disclosures of which are hereby incorporated by reference.

[0030] The molar ratio of ADH to dLOS in the reaction mixture is typically between about 10:1 and about 250:1. A molar excess of ADH is used to ensure more efficient coupling and to limit dLOS-dLOS coupling. In a preferred embodiment, the molar ratio is between about 50:1 and about 150:1; in a most preferred embodiment, the molar ratio is about 100:1. Similar ratios of AH-DLOS to both TT and HMP in the reaction mixture are also contemplated. In a preferred embodiment, one ADH per dLOS is present in the AH-DLOS conjugate. In another preferred embodiment, in the fmal dLOS-carrier protein conjugate, the molar ratio of DLOS to carrier is between about 15 and about 75, preferably between about 25 and about 50.

[0031] Immunogenicity of the conjugates in both mice and rabbits is enhanced by the use of monophosphoryl lipid A plus trehalose dimycolate (Ribi-700; Ribi Immunochemical Research, Hamilton, MT) as an adjuvant. Although this adjuvant is not approved for use in humans, the skilled artisan will appreciate that other well known standard adjuvants may be used in the invention, including aluminum compounds (i.e. alum), chemically-modified lipopolysaccharide, suspensions of killed *Bordetella pertussis*, N-acetylmuramyl-L-alanyl-D-glutamine and other adjuvants known to one of ordinary skill in the art. The use of aluminum compounds is particularly preferred. Such adjuvants are described by Warren et al. (*Ann. Rev. Biochem.*, 4:369-388, 1986), the entire disclosure of which is hereby incorporated by reference.

[0032] The dLOS-carrier protein conjugates for parenteral administration may be in the form of a sterile injectable preparation, such as a sterile injectable aqueous or oleaginous suspension. This suspension may be formulated according to methods well known in the art using suitable dispersing or wetting agents and suspending agents. The sterile injectable preparation may also be a sterile injectable solution or suspension in a parenterally acceptable diluent or solvent, such as a solution in 1,3-butanediol. Suitable diluents include, for example, water, Ringer's solution and isotonic sodium chloride solution. In addition, sterile fixed oils may be employed conventionally as a solvent or suspending medium. For this purpose, any bland fixed oil may be employed including synthetic mono- or diglycerides. In addition, fatty acids such as oleic acid may likewise be used in the preparation of injectable preparations.

[0033] The conjugate vaccine of the invention may be in soluble or microparticular form, or may be incorporated into microspheres or microvesicles, including liposomes. Although various routes of vaccine administration including, for example, intramuscular, subcutaneous, intraperitoneal and intraarterial are contemplated, the preferred route is intramuscular administration. In a preferred embodiment, the dosage of the conjugate administered will range from about 10 μg to about 50 μg. In a more preferred embodiment, the amount administered will be between about 20 μg and about 40 μg. In a most preferred embodiment, the amount administered is about 25 μg. Greater doses may be administered on the basis of body weight. The exact dosage can be determined by routine dose/response protocols known to one of ordinary skill in the art.

[0034] The vaccine of the invention may be administered to warm-blooded mammals of any age and are adapted to induce active immunization in young mammals, particularly humans, against otitis media and respiratory infections caused by NTHi. As a childhood vaccine, the conjugate is administered at about 2 to 4 months of age. Typically, two booster injections of between about 10 μg and about 25 μg are administered at about 2 and again about 13 months after the initial injection. Alternatively, three booster injections are given at 2, 4 and 16 months after the initial injection.

[0035] The IgG antibodies elicited by systemic administration of the conjugate vaccine will transfer to local mucosa and inactivate NTHI inoculum on mucosal surfaces (i.e., nasal passages). Secretory IgA will also play a role in mucosal immunity if the conjugate vaccine is administered to the mucosa (i.e. intranasally). Thus, the conjugate vaccine will prevent local, as well as systemic, NTHi infection.

[0036] The examples describe conjugate vaccines using NTHi strains 9274 and 2019. Vaccines from other NTMi strains are within the scope of the present invention and are made using the same techniques. NTHi strains 9274 and 2019 are classified as types III and II, respectively. Other clinically relevant NTHi strains contemplated as sources of dLOS for generation of a dLOS-carrier conjugate vaccine include strains 1479, 5657 and 7502 (type I, IV and V,

respectively). These strains, as well as strain 2019, are described by Campagnari et al. (*Infect. Immun.*, 55:882-887, 1987) and Patrick et al. (*Infect. Immun*, 55:2902-2911, 1987), the entire contents of which are hereby incorporated by reference, and are generally available from the research community.

[0037] A multivalent vaccine comprising a mixture of conjugates, each having a DLOS from a different NTHi strain, is also within the scope of the invention. A person of ordinary skill in the art will appreciate that LOS from these other clinically relevant strains may be detoxified by removal of fatty acids therefrom as described in Example 2. In a preferred embodiment, the dLOS moieties thus obtained are at least about 5,000 fold less toxic than LOS itself. In a particularly preferred embodiment, the dLOSs are at least about 10,000 fold less toxic than dLOS. Determination of toxicity may be performed, for example, according to Example 8 below.

[0038] NTHi 9274 and 2019 were grown and LOS was isolated as described in the following example.

EXAMPLE 1

Bacterial Growth and LOS Purification

[0039] NTHi strain 9274 was isolated from middle ear fluids of a patient with otitis media and provided by Dr. M .A. Apicella (University of Iowa, Iowa City, Iowa). Also see Gu et al., *Infect. Immun.*, 63:4115-4120, 1995. NTHi strain 2019 was isolated from chronic bronchitis patients (Campagnari et al., supra.). Bacteria were grown on chocolate agar plates in a 5% CO_2 atmosphere at 37° C. for 8 hours, then transferred to 200 ml 3% brain-heart-infusion (BilH) medium (Difco, Detroit, Mich.) containing 5 μg/ml NAD and 2 μg/ml hemin (Sigma, St. Louis, Mo.). The medium was placed in an incubator shaker set at 150 rpm and 37° C. overnight. The culture broth was transferred to five 2.8 1 flasks, each containing 1.4 liters BiH media Flasks were shaken at 140 rpm at 37° C. for 24 hours. The culture broth was centrifuged at 15,000×g for 30 minutes to separate cells and supernatant.

[0040] LOS was purified from cells by the classical phenol-water extraction method with modification (Westphal et al., *Meth. Carbohyd Chem.*, 5:83-91, 1965, incorporated by reference). Briefly, cells were grown overnight and treated with 90% phenol (45% fmal concentration) at 68-70° C. for 15-20 min., cooled on ice and centrifuged. After recovery of the upper aqueous phase, the remainder was re-extracted with water. Sodium acetate (5 mg/ml) was added to the combined aqueous phases and the LOS was precipitated with 2 volumes of acetone to reduce phospholipid contamination. The pellets were washed twice with 70% ethanol to reduce trace phenol, then dissolved in water. RNase and DNase were added (50-100 jig/ml) and samples were incubated at 37° C. for 3-5 hours. Proteinase K (0.5 mg/ml) was then added and the samples were incubated at 60° C. overnight, then centrifuged at least twice at 150,000×g for 3 hours. The gel-like LOS was dissolved in about 10 volumes of water and lyophilized.

[0041] LOS was also purified from the culture supernatant by gel filtration (Gu et al., *Anal. Biochem.*, 196:311-318, 1991, incorporated by reference). Briefly, 6-8 liters of culture supernatant were concentrated to 100-200 ml using a hollow-fiber cartridge with a 100,000 molecular weight cutoff (Amicon, Danvers, MA). To the concentrate was added 5-10 volumes of water, followed by reconcentration to about 400 ml. LOS containing outer membrane vesicles (OMV) were then pelleted by ultracentrifugation at 150,000×g and suspended in water to a 5-10% (v/v) suspension. To 10 ml of an opalescent OMV suspension, EDTA was added to a concentration of 2 mM, and the pH adjusted to 8.5 with 1 M NaOH. Sodium deoxycholate (Na-DOC) (2%, w/v) was added and the mixture was kept at 37° C. for 10 minutes to solubilize LOS from OMV. LOS was separated from outer membrane proteins on a 5×90 cm Sephacryl S-300 column (Pharmacia) using 20 mM Tris, pH 8.5, 2 mM EDTA, 1% Na-DOC, 0.02% sodium azide as an elution buffer. Proteins were monitored by absorbance at 280 nm.

[0042] LOS could be detected as ethanol precipitable materials as follows. To 200 μl aliquots of the column fractions, 3 volumes of ethanol (95%) were added after adjusting the aliquots to 0.25 M NaCl with 5 M NaCl. The mixtures were vortexed and LOS immediately precipitated. According to the LOS precipitation, the main LOS fractions were pooled, precipitated in 70% ethanol overnight as described above and collected by low speed centrifugation. LOS was dissolved in water to about 10 mg/ml for another cycle of ethanol precipitation to reduce residual NA-DOC, proteins and phospholipids, then washed with 70% ethanol, lyophilized and weighed.

[0043] The protein and nucleic acid content of purified LOS from both cells and culture supernatant was less than 1%.

[0044] The LOS purified from cells and from culture supernatant was combined and detoxified as described below.

EXAMPLE 2

Detoxification of LOS

[0045] LOS (160 mg), isolated as described in Example 1, was dried over phosphorus pentoxide (P_2O_5) for 3 days, suspended in 16 ml anhydrous hydrazine (Sigma) and incubated at 37° C. for 2 hours, mixing every 15 min. The solution was placed on ice, then added dropwise to cold acetone in an ice bath until a precipitate formed (>90% acetone). The mixture was centrifuged at 5,000×g at 5° C. for 30 min. The pellet was washed twice with cold acetone, dissolved in pyrogen-free water to 20 mg/ml, and centrifuged at 150,000×g for 3 hours at 5° C. The supernatant was lyophilized and applied to a 1.6×90 cm SEPHADEX G-50 gel filtration column (Pharmacia, Upssala, Sweden). The column was eluted with 25 mM ammonium acetate and monitored with a differential refractometer (R-400; Waters, Milford, Mass.). The eluate was assayed for carbohydrate content by the phenol-sulfuric acid method (Dubois et al., *Anal. Biochem.*, 28:250-256, 1956) and the carbohydrate fractions were pooled, lyophilized three times to remove the salt and designated dLOS.

[0046] After hydrazine treatment of LOS, the yield of dLOS ranged from 48% to 55% by weight for 9274 and 18% to 26% for 2019. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis of LOS and DLOS followed by silver staining showed that dLOS migrated faster than did LOS due to the removal of ester-linked fatty acids.

[0047] dLOS was derivatized with ADH as described in the following example.

EXAMPLE 3

Derivatization of dLOS

[0048] ADH was bound to the carboxyl group of the KDO moiety of dLOS to form AH-dLOS derivatives using EDC and N-hydroxysulfo-succinimide (sulfo-NHS) (Pierce, Rockford, Ill.). dLOS (70 mg) was dissolved in 7 ml 345 mM ADH (Aldrich, Milwaukee, Wis.) in water. The molar ratio of ADH to dLOS was about 100 to 1. Solid sulfo-NHS was added to a final concentration of 8 mM. The pH was adjusted to 4.8 with 1 M HCl and EDC was added to a concentration of 0.1 M. The reaction mixture was stirred and maintained at pH 4.8±0.2 with 1 M HCl for three hours at room temperature. The reaction mixture was adjusted to pH 7.0 and applied to the G-50 column.

[0049] The eluate containing AH-dLOS was assayed for carbohydrate (dLOS) content using dLOS as a standard, then calculated as moles using a molecular weight of 3,000. The amount of AH in AH-dLOS was measured by a modified TNBS method using ADH as a standard and measuring absorbance at OD_{490nm} (Kemp et al., *J. Immunol. Meth.*, 94:65-72, 1986). .

[0050] The peaks containing both carbohydrate and AH were pooled, lyophilized three times to remove the salt, and designated as AH-dLOS. AH-dLOS was measured for its composition using dLOS and ADH as standards. The final molar ratio of AH to dLOS in the AH-DLOS product=moles of AH/moles of DLOS. For strain 9274, the molar ratio of AH to DLOS was 0.47 and 0.55, indicating that about 50% of the LOS was derivatized. For strain 2019, the molar ratio of AH to dLOS was 0.37. The yields for strain 9274, on the basis of carbohydrate content, were 43% and 52% for the two lots of AH-dLOS. For strain 2019, the yield was 94%.

[0051] AH-dLOS was conjugated to TT as described in the following example.

EXAMPLE 4

Conjugation of AH-dLOS to Carrier Proteins

[0052] AH-dLOS was coupled to carboxyl groups on TT (strains 9274 and 2019) or HMPs (Strain 9274) at pH 5.6 with EDC. HMPs were purified from NTHi strain 12 as described by Barenkamp et al. (*Infect. Immun.*, 64:1246-1251, 1996). AH-dLOS (20 mg) was dissolved in 2 ml distilled water and mixed with 10 mg TT (5.9 mg/ml) (Connaught Labs, Inc., Swiftwater, Pa.) or 8 mg HMP (4 mg/ml). The molar ratio of AH-dLOS to TT and HMP was about 100 to 1. The pH was adjusted to 5.6 with 0.1 M HCl, followed by addition of EDC to a concentration of 0.1 M. The reaction mixture was stirred and maintained at pH 5.6±0.2 with 0.1 M HCl for 1 to 3 hours at room temperature. The reaction mixture was adjusted to pH 7.0, centrifuged at 1,000×g for 10 min and purified using a 1.6×90 cm SEPHACRYL® S-300 gel filtration column (Pharmacia) equilibrated with 0.9% NaCl. The eluate was monitored for protein by determining OD_{280} of the column fractions and assayed for carbohydrate content. Column fractions containing both protein and carbohydrate were pooled and designated as dLOS-TT or dLOS-HMP. Both conjugates were analyzed for carbohydrate and protein using dLOS and BSA as standards. For strain 9274, the molar ratio of dLOS to TT in the two lots of conjugate preparations was 26:1 and 50:1 and the yield was 10 to 15% (Table 1). For strain 2019, the molar ratio of dLOS to TT in the two lots of conjugate preparations was 31:1 and 42:1 and the yield was 18 to 26%.

TABLE 1A

Composition and yield of NTHi 9274 conjugates

Conjugate	dLOS (ug/ml)	protein (ug/ml)	Ratio of dLOS to protein[a] (mol of dLOS/ mol of protein)	Yield[b] (%)	A_{405} (hyper-immune serum/MAb)
dLOS-TT1	100	190	26	15	1.56/1.49
dLOS-TT2	90	90	50	11	1.50/1.38
dLOS-HMP	235	274	34	15	1.53/1.45

[a]The ratio is expressed as moles of dLOS per mole of protein using molecular weight of 3,000 for dLOS, 150,000 for TT, and 120,000 for HMP.
[b]Based on the starting amounts of dLOS and dLOS contained in the conjugates.
[c]The antigenicity of conjugates was expressed as ELISA reactivity at A_{405} when the conjugates were used as coating antigens (10 μg/ml) and the mouse hyperimmune sera (1/500) and MAb 6245B4 (1/1000) were used as binding antibodies. LOS (10 μg/ml) also showed A_{405} values of 1.02 and 1.10 under the same conditions.

[0053]

TABLE 1B

Composition, yield and antigenicity of NTHi 2019 conjugates

Conjugate	Amt (ug/ml) of:[a] dLOS	TT	Molar ratio dLOS to TT	Yield (%)	A_{405}[c] (hyperimmune serum)
dLOS-TT1	238	380	31	18	1.4
dLOS-TT2	280	340	42	26	1.2

[a]The ratio is expressed as moles of dLOS per mole of protein with molecular weights of 3,000 for dLOS and 150,000 for TT.
[b]Based on the starting amount of dLOS and the dLOS contained in the conjugates as measured by the phenol-sulfuric acid method.
[c]The antigenicity of conjugates was expressed as ELISA reactivity at A_{405} when the conjugates were used as coating antigens (10 ug/ml) and a rabbit hyperimmune serum (1/4,000) was used as a binding antibody. LOS (10 ug/ml) also showed an A_{405} value of 1.7 under the same conditions.

[0054] The antigenicity of the conjugates was determined as described below.

EXAMPLE 5

Antigenicity of dLOS, AH-dLOS and dLOS-TT

[0055] The antigenicity of dLOS, AH-dLOS and the dLOS conjugates was tested by double immunodiffusion and/or enzyme-linked immunosorbent assay (ELISA) using hyperimmune serum to NTHi 9274 or 2019 whole cells and a mouse monoclonal antibody (6245B4) generated against NTHi 9274 LOS. Hyperimmune serum was produced by intraperitoneally injecting 10 Balb/c mice three times at two week intervals with about 10^9 bacteria per injection. Blood samples were collected one week after the third injection.

[0056] Double immunodiffusion was performed in 0.8% agarose in phosphate-buffered saline (PBS, pH 7.4). Both AH-dLOS and the dLOS conjugates bound to the LOS

MAb. dLOS-TT and LOS formed identical precipitation lines in double immunodifflusion. Both showed comparable binding to the LOS MAb and hyperimmune sera by ELISA, indicating that dLOS retained the LOS antigenicity. dLOS-HMP exhibited similar results to those of DLOS-TT.

[0057] For ELISA, a 100 ii sample of conjugates at 10 μg dLOS/ml in PBS, or LOS at 10 μg/ml in PBS containing 10 mM $MgCl_2$ was used to coat microwell plates overnight (Immuno I plate; Dynatech Laboratories, Inc., Alexandria, Va.). The plate was blocked with 150 μl 1% BSA in PBS for 1 hour, followed by addition of 100 μl diluted mouse serum or MAb and incubation for 3 hours. Alkaline phosphatase-conjugated goat anti-mouse IgG or IgM (Sigma) was then added followed by incubation for an additional 2 hours. Diluents for sera and phosphatase were 1% BSA in PBS containing 0.05% Tween-20 (pH 7.4). All steps were performed at room temperature and, between each step, the wells were washed five times with 0.9% NaCl containing 0.05% Tween-20 (pH 7.4). The enzyme substrate was added and the plates were read with a microplate autoreader set at OD_{405nm} (EL309, Bio-tek Instruments). The conjugates showed an ELISA binding reactivity comparable to that of LOS with both LOS MAb and immune sera to whole NTHi 9274 or 2019 cells.

[0058] The immunogenicity of the NTHi 9274 DLOS-TT conjugates was determined as described in the following example.

EXAMPLE 6

Immunogenicity of NTHi 9274 dLOS-TT conjugates

[0059] The immunogenicity of the NTHi 9274 dLOS-TT conjugates was tested in both mice and rabbits. Five week old general purpose mice (NIH/Swiss, female), ten mice per group, were subcutaneously immunized with 5 μg of the conjugates (based on carbohydrate weight), LOS, DLOS, dLOS plus TT (10 μg) or TT only in 0.2 ml 0.9% NaCl with or without Ribi-700 adjuvant containing 50 μg monophosphoryl lipid A and 50 μg synthetic trehalose dimycolate. Mice were injected three times at three week intervals and bled 14 days after the first injection and seven days after the second and third injections.

[0060] New Zealand white rabbits (female, 2-3 kg), 2-3 rabbits per group, were subcutaneously immunized with 50 μg DLOS or conjugates (carbohydrate weight) in 1 ml 0.9% NaCl with or without Ribi-700 adjuvant. Rabbits were injected twice at one-month intervals and bled two weeks after the first injection and 11-14 days after the second injection.

[0061] Serum anti-LOS levels were expressed in ELISA units, using NTHi 9274 LOS as a coating antigen and the NTHi 9274 hyperimmune serum as a reference assigned values of 4,000 and 3,500 units/ml for IgG and IgM, respectively. Pre-sera contained five (3-9) units of IgG and three (1-6) units of IgM. Serum TT antibody was measured by ELISA in which TT (5 Ξg/ml) was used as a coating antigen and a horse anti-TT serum (20 IU/ml) as a reference assigned a value of 320 ELISA units/ml for IgG and IgM, respectively. Serum HMP antibody was measured by ELISA in which HMP (5 μg/ml) was used as a coating antigen, and expressed as ELISA units on the basis of a reference mouse serum produced by three injections of HMP and assigned values of 2,000 and 10 ELISA units/ml for IgG and IgM, respectively.

[0062] Antibody levels are expressed as the geometric mean in ELISA units of n independent observations ±standard deviation or range (n<4). Significance was tested with the two-sided T-test and P values <0.05 were considered significant.

[0063] In mice, dLOS or a mixture of dLOS and TT (unconjugated) did not elicit LOS antibodies. The antibody response to LOS elicited by the conjugates is summarized in Table 2. dLOS-TT elicited low LOS IgG levels after the first injection which increased 28 to 162-fold after the second and third injections (P <0.01). dLOS-HMP elicited significant IgG levels after the first injection which also increased after the second and third injections (104 to 486-fold, P <0.01). After 3 injections, dLOS-HMP elicited higher IgG levels than did DLOS-TTI (P <0.01) or dLOS-TT2 (P<0.05). LOS alone elicited low IgG levels after the first injection which increased 25 to 84-fold after the second and third injections (P<0.05).

[0064] The immunogenicity of the conjugates was significantly enhanced by Ribi adjuvant. One dose elicited comparable or higher IgG levels than did two doses of the conjugates alone. After three injections, about a five-fold increase in IgG was observed after three injections (P<0.01).

[0065] The conjugates elicited low to medium levels of IgM after each injection. LOS elicited high IgM levels after the first injection which increased after the second and third injections (P<0.01). Ribi adjuvant enhanced IgM levels in the conjugate groups.

[0066] As set forth herein, "adjuvant" is Ribi-700 adjuvant.

TABLE 2

Murine antibody response to NTHi 9274 LOS elicited by conjugates

Immunogen	Injection No.	Geometric mean ± SD ELISA units IgG	IgM
dLOS	1	5 (3–9)	5 (3–8)
	2	6 (3–10)	3 (1–5)
	3	4 (3–7)	2 (1–3)
dLOS-TT1	1	8 (5–13) +*	14 (8–25)
	2	140 (55–352)++	42 (7–237)
	3	217 (91–516)	52 (11–251)
dLOS-TT2	1	10 (3–36)+	17 (4–84)
	2	217 (47–1,007)++	42 (7–237)
	3	810 (229–2,890)	42 (24–74)
dLOS-TT2 + adjuvant	1	270 (62–1,168)*a	470 (266–828)
	2	1,257 (313–5,045)**	101 (64–160)
	3	4,698 (2,664–8,284)	81 (39–169)
dLOS-HMP	1	72 (45–115)+	11 (7–18)
	2	522 (296–920)++	101 (64–160)
	3	2,430 (1,168–5,055)	14 (8–25)
dLOS-HMP + adjuvant	1	1,403 (780–2,524)+	243 (91–516)
	2	7,290 (2,252–23,593)++	421 (234–757)
	3	11,006 (6,233–19,435)	19 (11–33)
dLOS + TT	1	4 (3–7)	2 (2–4)
	2	4 (3–5)	3 (1–6)
	3	4 (3–7)	3 (1–11)
LOS	1	65 (38–110)*	195 (123–310)
	2	125 (60–263)**	338 (161–709)
	3	419 (238–739)	470 (186–1,186)

** versus **, P < 0.05 and + versus ++, P < 0.01.

[0067] The murine antibody response to TT elicited by the conjugates is shown in Table 3. dLOS did not elicit antibodies to TT. However, both lots of dLOS-TT elicited IgG antibodies to TT after the first injection which rose significantly after the second and third injections (P<0.01). Either TT alone, or the mixture of TT and dLOS resulted in enhanced IgG responses after two or three injections compared to the conjugates (P<0.01 or <0.05). Ribi adjuvant enhanced the levels of IgG in the conjugate groups.

[0068] The conjugates, TT or the unconjugated mixture of TT and dLOS elicited low IgM levels after each injection. Ribi adjuvant enhanced the levels of IgM in the conjugate groups.

TABLE 3

Murine antibody response to tetanus toxoid elicited by dLOS-TT conjugates

Immunogen	Injection No.	Geometric mean ± SD ELISA units IgG	IgM
dLOS	1	<1	<3
	2	<1	<3
	3	<1	<3
dLOS-TT1	1	30 (14–62)*•	16 (9–27)
	2	112 (29–433)••	37 (12–116)
	3	419 (238–739)	12 (8–20)
dLOS-TT2	1	8 (3–25)+*	12 (8–20)
	2	90 (17–463)++	19 (8–49)
	3	522 (296–920)	4 (3–7)
dLOS-TT2 + adjuvant	1	72 (13–397)+	30 (14–50)
	2	1,257 (713–2,217)++	72 (45–115)
	3	1,257 (713–2,217)	112 (71–178)
dLOS + TT	1	37 (24–59)+	4 (3–7)
	2	1,257 (713–2,217)++	12 (8–20)
	3	2,430 (863–6,846)	19 (8–49)
TT	1	24 (15–38)+	4 (3–7)
	2	650 (409–1,033)++	12 (5–30)
	3	1,951 (505–7,528)	19 (4–93)

*• versus ••, P < 0.05 and + versus ++, P < 0.01.

[0069] dLOS itself did not elicit anti-HNT antibodies. dLOS-HMP elicited low IgG levels after the first injection which rose significantly after the second and third injections (P<0.01). HMP alone showed similar results to dLOS-HMP and no significant differences in IgG levels between the two groups were observed after 3 injections (P>0.05). IgG levels were significantly increased by inclusion of adjuvant in the conjugate group (P<0.01). DLOS-HMP or HMP alone elicited low IgM levels after each injection. Ribi adjuvant enhanced IgM levels elicited by DLOS-HMP which increased significantly after each injection (P<0.01). The results are summarized in Table 4.

TABLE 4

Murine antibody response to high molecular weight proteins of NTHi elicited by dLOS-HMP conjugates

Immunogen	Injection No.	Geometric mean ± SD ELISA units IgG	IgM
dLOS	1	<1	<3
	2	<1	<3
	3	<1	<3
dLOS-HMP	1	2 (1–3)+*	6 (3–12)
	2	243 (117–506)++	12 (8–20)
	3	1,756 (732–4,134)	8 (3–19)
dLOS-HMP +	1	243 (123–490)	5 (3–10)

TABLE 4-continued

Murine antibody response to high molecular weight proteins of NTHi elicited by dLOS-HMP conjugates

Immunogen	Injection No.	Geometric mean ± SD ELISA units IgG	IgM
adjuvant	2	2,187 (1,096–4,365)	52 (29–93)
	3	>6,561	130 (74–229)
HMP	1	1	3
	2	729 (205–2,592)	5 (3–10)
	3	1,516 (860–2,674)	7 (4–12)

*+ versus ++, P < 0.01.

[0070] Two to three rabbits for each group were subcutaneously immunized on days 0 and 28 with 50 μg dLOS, conjugates or conjugates plus Ribi adjuvant. Blood samples were collected on days 0, 14 and 38-42. DLOS itself did not elicit anti-LOS antibodies in rabbits. DLOS-TT elicited a significant increase in IgG levels after the first and second injections (39 to 168-fold, P<0.01). The results for DLOS-HMP were similar to those observed for dLOS-TT (27 to 243-fold). Ribi adjuvant enhanced IgG levels elicited by dLOS-TT (P<0.05), but not by dLOS-HMP, after the second injections. Both conjugates, either alone or with adjuvant, elicited low IgM levels after each injection. Pre-sera contained 1 unit for IgG and <1 unit for IgM. The results are summarized in Table 5.

TABLE 5

Rabbit antibody response to NTHi 9274 LOS elicited by conjugates

Immunogen	Injection No.	Geometric mean ± SD ELISA units IgG	IgM
dLOS	1	1	<1
	2	1	<1
dLOS-TT	1	39 (27–81)*•	9
	2	168 (81–243)••	9
dLOS-TT + adjuvant	1	27	5 (3–9)
	2	421 (243–729)++*	16 (9–27)
dLOS-HMP	1	27	3
	2	243	9
dLOS-HMP + adjuvant	1	81	9
	2	243	9

*• versus ••, P < 0.05 and + versus ++, P < 0.01.

[0071] dLOS itself did not elicit TT antibodies in rabbits. dLOS-TT elicited anti-TT IgG after the first injection which rose significantly after the second injection. IgG levels were enhanced by adjuvant 3-fold after the second injection. Both conjugates, either alone or with adjuvant, elicited low IgM levels after each injection. The results are summarized in Table 6.

TABLE 6

Rabbit antibody response to tetanus toxoid elicited by dLOS-TT conjugates

Immunogen	Injection No.	Geometric mean ± SD ELISA units IgG	IgM
dLOS	1	<3	1
	2	<3	1
dLOS-TT	1	19 (9–27)	9
	2	729	13 (9–27)

TABLE 6-continued

Rabbit antibody response to tetanus toxoid elicited by dLOS-TT conjugates

Immunogen	Injection No.	Geometric mean ± SD ELISA units IgG	IgM
dLOS-TT2 + adjuvant	1	16 (9–27)	3
	2	2,187	5 (3–9)

[0072] dLOS itself did not elicit HMP antibodies in rabbits. dLOS-HMP elicited anti-HMP IgG after the first injection which rose significantly after the second injection. IgG levels were enhanced by adjuvant 3-fold after the second injection. Both conjugates, either alone or with adjuvant, elicited low IgM levels after each injection. The results are summarized in Table 7.

TABLE 7

Rabbit antibody response to high molecular weight proteins elicited by dLOS-HMP conjugates

Immunogen	Injection No.	Geometric mean ± SD ELISA units IgG	IgM
dLOS	1	<3	1
	2	<3	1
dLOS-HMP	1	16 (9–27)+[a]	3
	2	421 (243–729)++	3
dLOS-HMP + adjuvant	1	27	3
	2	1,263 (729–2,187)+++	3

[a]+ versus ++, P < 0.01 and ++ versus +++, P = 0.0519.

EXAMPLE 7

Immunogenicity of NTHi 2019 dLOS-TT Conjugates

[0073] Serum anti-LOS levels were expressed in ELISA units, using NTHi 2019 LOS as a coating antigen and the NTHi 2019 hyperimmune serum as a reference assigned values of 4,000 and 3,500 units/ml for IgG and IgM, respectively. Pre-sera contained five (3-9) units of IgG and three (1-6) units of IgM. Serum TT antibody was measured by ELISA in which TT (5 jig/ml) was used as a coating antigen and a horse anti-TT serum (20 IU/ml) as a reference assigned a value of 320 ELISA units/ml for IgG and IgM, respectively.

[0074] Antibody levels are expressed as the geometric mean in ELISA units of n independent observations ±standard deviation or range (n<4). Significance was tested with the two-sided T-test and P values <0.05 were considered significant.

[0075] The immunogenicity of the NTHi 2019 dLOS-TT conjugates was tested in both rabbits and mice. New Zealand white rabbits (female, 2-3 kg), 1-3 rabbits per group, were subcutaneously and intramuscularly immunized with 50 μg dLOS, dLOS-TT1, DLOS-TT_1 with Ribi adjuvant or LOS (carbohydrate weight) in 1 ml 0.9% NaCl. Rabbits were injected three times at one-month intervals and bled 0 and 10-14 days after the first, second and third injections; 1, 3, and 6 months after the third injection; and 10 days after the fourth injection.

[0076] dLOS itself did not elicit anti-LOS antibodies in rabbits. dLOS-TT elicited a significant increase in IgG levels after the second and third injections (40 to 58-fold, P<0.01). Ribi adjuvant significantly enhanced IgG levels elicited by dLOS-TT (P<0.05) after the second injection. Pre-sera contained 1 unit for IgG and <1 unit for IgM. The results are summarized in Table 8.

TABLE 8

Rabbit antibody response to NTHi 2019 LOS elicited by dLOS-TT

Immunogen units[a]	Bleeding no.[b]	IgG Geometric mean (range) ELISA units
dLOS	1	3
	2	3
	3	3
	4	3
dLOS-TT1	1	1.4 (1–3)
	2	3
	3	56 (27–81)
	4	81 (27–243)
	5	39 (27–81)
	6	19 (9–27)
	7	9
	8	81
dLOS-TT1 + adjuvant	1	1
	2	16 (9–27)
	3	729
	4	1,263 (729–2187)
	5	729
	6	421 (243–729)
	7	243 (81–729)
	8	1,263
LOS	1	3
	2	3
	3	3
	4	3

[a]One to three rabbits for each group were subcutaneously and intramuscularly immunized on 0, 1, 2, and 6 months with 5 ug of dLOS, dLOS-TT, dLOS-TT with Ribi adjuvant, or LOS.
[b]Blood samples were collected on 0, 10–14 days after the 1st, 2nd, and 3rd injeciton (2–4), 1, 3, and 6 months after the 3rd injection (5–7), and 10 days after the 4th injection (8).

[0077] dLOS itself did not elicit TT antibodies in rabbits. dLOS-TT elicited anti-TT IgG after the first injection which rose significantly after the second injection. IgG levels were enhanced by adjuvant 3-fold after the second injection. The results are summarized in Table 9.

TABLE 9

Rabbit antibody response to TT elicited by dLOS-TT from NTHi 2019

Immunogen units[a]	Bleeding no.[b]	IgG Geometric mean (range) ELISA units
dLOS (or LOS)	1	1
	2	1
	3	1
	4	1
dLOS-TT1	1	1.4 (1–3)
	2	8 (5–10)
	3	810
	4	1,168 (810–2,430)
	5	810
	6	270
	7	130 (90–270)
	8	810
dLOS-TT1 + adjuvant	1	1
	2	30
	3	2,430

TABLE 9-continued

Rabbit antibody response to TT elicited by dLOS-TT from NTHi 2019

Immunogen units[a]	Bleeding no.[b]	IgG Geometric mean (range) ELISA units
	4	4,209 (2,430–7,290)
	5	1,403 (810–2,430)
	6	468 (270–810)
	7	156 (90–270)
	8	4,209

a, b: See Table 8.

[0078] Female Balb/c mice (10-20 mice per group), were subcutaneously immunized with 5 μg (based on carbohydrate weight) of dLOS, dLOS-TT_1, DLOS-TT with Ribi adjuvant or LOS in 0.2 ml 0.9% NaCl. Mice were injected three times at three week intervals and bled 14 days after the first injection, seven days after the second and third injections and 1 week, 1 month and 5 months after the third injection.

[0079] dLOS-TT did not elicit antibodies against dLOS, and elicited a weak antibody response against LOS. Immunogenicity of the conjugate was increased four fold after the first injection and 14 fold after the second injection. The response was not greatly enhanced by adjuvant after the first and second injections; however, higher levels of IgG were maintained after injections 3, 4, and 5 in the presence of adjuvant. The results are summarized in Table 10.

TABLE 10

Murine antibody response to NTHi 2019 LOS elicited by dLOS-TT

Immunogen units[a]	Bleeding no.[b]	IgG Geometric mean (range) ELISA units
dLOS	1	4 (2–8)
	2	3
	3	3
dLOS-TT1	1	11 (7–18)
	2	42 (12–147)
	3	157 (86–286)
	4	52 (12–228)
	5	14 (8–25)
dLOS-TT1 + adjuvant	1	6 (2–16)
	2	42 (16–112)
	3	101 (24–423)
	4	101 (20–515)
	5	157 (45–548)
LOS	1	6 (3–12)
	2	9
	3	9

[a]Ten to twenty mice for each group were given a total of three subcutaneous injections at 3-week intervals with 5 ug of dLOS, dLOS-TT, dLOS-TT with Ribi adjuvant, or LOS.
[b]Blood samples were collected 2 weeks after the 1st injection, 1 week after the 2nd injection, and 1 week, 1 month, and 5 months after the 3rd injection (3–5).

[0080] To determine the residual endotoxin reactivity of dLOS, both the limulus amebocyte lysate (LAL) assay and mouse lethal toxicity assays were used as described in the following two examples.

EXAMPLE 8

Limulus Amebocyte Assay and Rabbit Pyrogen Test

[0081] LOS, DLOS and DLOS conjugates were diluted with pyrogen-free water. Equal volumes (100 μl) of samples and Limulus amebocyte lysate were mixed and incubated at 37° C. for 1 hour. Gelation of the lysate at the minimal LOS concentration was determined by inverting the mixture. A formn gel was considered a positive reaction (Hochstein et al., *Bull. Parenteral Drug Assoc.*, 27:139-148, 1973). All reagents were from the U.S. Food and Drug Administration, Bethesda, Md. The sensitivity of the LAL assay is 0.09 EU per ml. By the LAL assay, the LOS from strain 9274 had the LAL reactivity at 10,000 EU/gg, while the dLOS exhibited this reactivity at 1 EU/μg. Thus, dLOS was 10,000 fold less active than untreated LOS in promoting LAL gelation. For the LOS from strain 2019, the LAL reactivity was 10,000 EU/μg, while the dLOS exhibited this reeactivity at 0.5 EU/μg. This represents a 2×10,000-fold reduction in toxicity of DLOS compared to LOS.

[0082] Pyrogenicity of the strain 9274 dLOS-TT conjugate in rabbits was assayed by H. D. Hochstein, U.S. FDA. dLOS-TT conjugate (25 μg) was intravenously injected into each rabbit, assuming this is one dose for human use. The conjugate was not pyrogenic in rabbits when injected at 8-9 μg/kg of rabbit body weight. In comparison, the World Health Organization (WHO) specification for Hib polysaccharide conjugate vaccines is to be nonpyrogenic at 1 μg/kg of rabbit body weight. The conjugates were also assayed for general safety by H. D. Hochstein and were deemed acceptable by the requirements of 21 C.F.RP § 610.11.

EXAMPLE 9

Mouse Lethal Toxicity Test

[0083] LOS and dLOS from strain 9274 were tested by a mouse lethality assay (Galanos et al., *Proc. Natl. Acad. Sci. USA*, 76:5939-5943, 1979). Briefly, female seven week old inbred BALB/c mice, 8 per group, were injected intraperitoneally with 8 mg D-galactosamine HCl (Sigma) dissolved in 0.2 ml pyrogen-free water (400 mg/kg). Within 30 min., the animals were given different amounts of the experimental preparations in 0.2 ml water by intravenous route. Lethality was observed over a four day period and the LD_{50} was calculated.

[0084] The LD_{50} of dLOS was 18.3 μg, while the LD_{50} of LOS was 0.3 ng. Thus, dLOS was at least 10,000 fold less toxic than untreated LOS.

EXAMPLE 10

Bactericidal Assay of dLOS-TT Rabbit Antisera

[0085] Bactericidal activity of DLOS-TT antisera was based on a modification of a microbactericidal assay (Frasch et al., *J Exp. Med*, 147:629-644, 1978; Jennings et al., *J. Exp. Med.*, 165:1207-1221, 1987), the entire contents of which are hereby incorporated by reference. Rabbit preimmune and postimmune sera obtained after two injections of conjugate were inactivated at 56° C. for 30 min. and tested for bactericidal activity against NTHi strain 9274, the prototype strain 3198 (Type III) and strain NTHi 2019. Briefly, serial

twofold dilutions of the sera were made in Dulbecco's PBS containing calcium, magnesium, and 0.1% gelatin (DPBSG), such that 50 μl sera or diluted sera were present in each well of a sterile 96-well plate. NTHi strains were grown on chocolate agar plates at 37° C., 5% CO_2 overnight and 3-5 colonies were transferred to another plate and incubated for 4.5 hours. Bacteria were diluted in DPBSG, and 30 μl bacterial suspension (about 3×103 CFU/ml) were added to each well. Infant rabbit serum (15 μl/well) was added as a source of complement (Pel-Freez, Brown Deer, Wis.) for strains 9274 and 3198. Guinea pig serum (Sigma) was used as a source of complement for strain 2019. Plates were incubated at 37° C. for 30 min. Fifty μl of the mixture was removed from each well and spread on chocolate agar plates (100×15 mm) which were then incubated at 37° C., 5% CO_2 overnight and colonies were counted. Controls included complement, inactive complement and a positive serum. The results are shown in Tables 11 (NTHi 9274) and 12 (NTHi 2019).

TABLE 11

Bactericidal activity of NTHi 9274 dLOS and dLOS conjugates

Rabbit		Bactericidal titer		Geometric Mean Titer + SD	
No.	Immunogen	pre-	post-sera	pre-	post-sera
1136	dLOS	2	2	2	2
1135	dLOS-TT	4	64		
104	dLOS-TT	0	32	1	51 (35-76)
108	dLOS-TT	0	64		
1139	dLOS-TT + Ribi	0	32	2	64 (24-171)
105	dLOS-TT + Ribi	4	128		
1	dLOS-HMP	8	128	4	64 (24-171)
2	dLOS-HMP	2	32		
3	dLOS-HMP + Ribi	2	64	2	64

[0086]

TABLE 12

Bactericidal activity of NTHi 2019 dLOS and dLOS conjugates

Immunogen[a]	Rabbit number[b]	Bactericidal titer[c]
dLOS	2005	<2
dLOS-TT	110	<2
	113	2
	1137	<2
dLOS-TT +	1131	2
adjuvant	1132	8
LOS	2004	<2

[a]See Table 8, footnote.
[b]Pre- and postimmune sera obtained after three injections were used.
[c]Expressed as the fold increase above the value for preimmune sera and given as the reciprocal of the serum dilution causing >50% killing of the bacteria.

[0087] The highest serum dilution causing a >50% killing was expressed as the reciprocal bactericidal titer. The immune sera in the presence or absence of adjuvant exhibited bactericidal activity against NTHi strain 9274 with mean titers of 1:51 and 1:64 (FIG. 1). There was a positive correlation between LOS IgG antibody levels and bactericidal titers (r=0.81, P<0.01). The immune sera also exhibited bactericidal activity against the LOS prototype strain 3198 (type III) with titers ranging from 1:2 to 1:16.

EXAMPLE 11

Binding Reactivities of dLOS-TT to NTHi Clinical Isolates

[0088] The reactivities of rabbit immune sera elicited by the dLOS-TT conjugates from NTHi 9274 and 2019 were analyzed by a whole cell ELISA using 155 NTHi clinical isolate strains. By whole cell ELISA, rabbit sera elicited by dLOS-TT conjugates from strain 9274 showed binding reactivities to 60-81% of U.S. strains and 45-80% of Japanese strains. Rabbit sera elicited by conjugates from strain 2019 showed binding reactivities to 21-57% of U.S. strains and 20-55% of Japanese strains. Combined sera elicited by strains 9274 and 2019 showed binding reactivities to 78-86% of U.S. strains and 75-93% of Japanese strains. The results are summarized in Table 13.

TABLE 13

Binding reactivities of rabbit immunosera elicited by conjugates from NTHi strains 9274 and 2019

	ELISA binding reactivities using 155 clinical isolates % (P/T)	
Immunosera	USA	Japan
1. dLOS-TT (9274)	81 (81/100)	80 (44/55)
2. dLOS-TT (9274) + adjuvant	60 (60/100)	60 (33/55)
3. dLOS-HMP (9274)	60 (60/100)	45 (25/55)
4. dLOS-HMP (9274) + adjuvant	62 (62/100)	45 (25/55)
5. dLOS-TT (2019)	57 (57/100)	55 (30/55)
6. dLOS-TT (2019) + adjuvant	21 (21/100)	20 (11/55)
5 plus 1	86 (86/100)	93 (51/55)
5 plus 2	81 (81/100)	82 (45/55)
5 plus 3	78 (78/100)	75 (41/55)
5 plus 4	80 (80/100)	78 (43/55)

[0089] The following study examines the ability of the dLOS-TT conjugate to protect against NTHi infection in a chinchilla model of otitis media This was a randomized, blind, controlled study of active prevention of acute otitis media caused by NTHi in chinchillas which was approved by NINDS/NIDCD ACUC, National Institutes of Health.

[0090] Experimental Scheme

[0091] A total of 58 healthy adult chinchillas, weighing between 400 and 600 grams each, were obtained from an outbred ranch (Moulton Chinchilla Ranch, Rochester, MN) and housed in separate cages throughout the experiments. All animals were quarantined for one week to acclimate to the laboratory prior to treatment. The animals were randomly assigned to three groups: 1) Control; 2) DLOS-TT; and 3) dLOS-HMP (FIG. 2). A blood sample was obtained from the transverse venous sinus of each chinchilla (Boellcher et al., *Lab. Anim. Sc.*, 40:223-224, 1990). Three days after bleeding, the animals were immunized with three doses of the coded vaccines or saline (control) at four week intervals and challenged with 140 colony forming units (CFU) of NTHi strain 9274 into the right middle ear 14 days after the last immunization (FIG. 2). Both ears were examined daily by otoscopy for evidence of acute otitis media during a period of 21 days after challenge. On day 3, 7, 14 and 21 post-challenge, the animals were sacrificed by overdose ketamine injection and cervical dislocation, and the middle ear fluids (MEFs) from both sides of ears were taken out and cultured for bacterial counting. Blood samples were

also collected from all chinchillas 14 days after the first and second immunization, 10 days after the third immunization and before sacrifice. The animals were anaesthetized with ketamine-HCl (30 mg/kg of body weight, im) prior to all operative procedures.

EXAMPLE 12

Immunization and Middle Ear Challenge

[0092] Animals were immunized with 25 μg NTHi 9274 dLOS-TT, dLOS-HMP (dLOS content) or saline in a total volume of 0.3 ml (0.15 ml intramuscularly in the right rear leg and 0.15 ml subcutaneously in the back) for each time (**FIG. 2**). Conjugates were prepared as described in the previous examples. The composition of dLOS-TT was 150 μg/ml of dLOS and 231 μg/ml of TT with the molar ratio of DLOS to TT at 32:1, while the composition of dLOS-HMP was 152 μg/ml of DLOS and 223 μg/ml with the molar ratio at 25.5:1. All immunogens were emulsified 1:1 in complete Freund's adjuvant (CFA) for the first and in incomplete Freund's adjuvant (IFA) (Difco, Detroit, Mich.) for the second and third immunizations.

[0093] NTII strain 9274 bacteria were recovered from Greave's solution stocks by transfer of a loopful of thawed organisms to a chocolate agar plate and incubated at 37° C. under 5% CO_2 for 16 hours. The next day, 5-10 colonies were transferred to 50 ml of 3% brain-heart infusion (BHI) broth with NAD (5 μg/ml) and hemin (2 μg/ml) (Sigma) in a 250 ml Erlenmeyer flask. Cells were grown for 4-6 hours at 37° C. in a shaker incubator (Model G25, New Brunswick Scientific Co., Edison, N.J.) at 150 rpm. Bacteria in mid-log growth phase (OD_{600}=0.5-0.6) were harvested by centrifugation (4,000×g) at 4° C. for 10 minutes, then washed twice with PBS containing 0.5% BSA, 0.15 mM $CaCl_2$ and 0.5 mM $MgCl_2$ (Barenkamp, *Infect. Immun.*, 64:1246-1251, 1996). The washed bacteria were maintained at 4° C. prior to animal challenge.

[0094] After anaesthetization, the area over the superior bulla of each animal was shaved and cleaned. Under sterile conditions, 0.2 ml of approximately 140 CFU bacteria was directly injected into the right middle ear via puncture of the superior aspect of the right cephalic bulla (Giebink, "The experimental otitis media due to Haemophilus influenzae in the chinchilla", in Haemophilus influenzae, Sell et al., Eds., Elsevier, New York, pp. 73-80, 1982; DeMaria et al., *Infect. Immun.*, 64:5187-5192, 1996, both incorporated by reference) with a 25-gauge needle attached to a 1 ml syringe. This inoculum was predetermined to induce otitis media within 48 hours.

EXAMPLE 13

Otoscope Observations

[0095] Animals were examined daily by two operators with otoscope postchallenge. A diagnosis of acute otitis media was made by the linear criterion rating from 0 to 4 scale of middle ear pathology based on the methods of Giebink et al. (*Laryngoscope*, 93:208-211, 1983) and Green et al. (*Meth. Enzymol.*, 235:59-68, 1994), both of which are hereby incorporated by reference, with modifications. A rating of 0 was a normal tympanic membrane (TM) with no pathological change around. A "1" was given if any congestive on the edges of TM was shown but TM was normal. A "2" was rated when severe congestive TM, opaque TM or any indication of minimal pathological changes was present, but TM can be moved by aspiration with the tubulerating. A "3" was assigned when there was evidence of moderate pathological changes, bulging TM, erythema, but no observable air-fluid level or fluids were seen. A "4" was given if any observable air-fluid level, yellow or bloody fluid was seen or TM cannot be moved, or perforation and discharge or substantial effusion volume with signs of severe inflammation were observed. The diagnosis of inner ear infection was made when the animal had auditory vertigo (Shurin et al., *J Pediatr.*, 97:364-369, 1980; Giebink et al., supra.).

[0096] On days 3, 7, 14, and 21 postchallenge, 4-5 chinchillas in each group were sacrificed (**FIG. 2**) and the superior bullae were opened. MEFs were aspirated from inferior bullae by a 23-gauge needle with suitable angle, then the middle ears were washed with 0.5 ml sterile PBS. The volume, color and consistency of MEFs were recorded. An aliquot of MEFs and their serial dilutions was plated into chocolate agar for a quantitative count of recovered NTHi. All procedures were performed under sterile conditions.

[0097] All control animals developed NTHi otitis media with effusion from one or both sides of the ears up to 21 days postchallenge. However, in the group administered the conjugate, only 60%, 80% and 60% developed NTHi otitis media with efflusion on day 3, 7, and 14, respectively (**FIG. 3**). On day 21, none of the animals in the dLOS-TT group and only 50% of the animals in the dLOS-HMP group showed otitis media with effusion. The numbers of otitis media with effusion were reduced 78% on day 21 (2/9 vs. 4/4; p=0.02) and 44% on whole course (22/39 vs. 19/19; p=0.0003) in the conjugate group compared to the control group.

[0098] The incidence of NTHi culture-positive MEFs from both ears of each group on days 3, 7, 14 and 21 postchallenge is shown in Table 14. The incidence of NTHi culture positive MEFs from total ears was reduced 44% by day 3 (p=0.07), 85% by day 21 (p=0.003), and 46% during the whole course (p=0.0003) in conjugate groups compared to the control group. The incidence of NTHi culture positive MEFs from the left (unchallenged) ear dropped 100% by day 21 (p=0.077), and 51% during the whole course (p=0.04) in the conjugate group relative to the control group. The results from the right ear (challenged ear) were consistent with those of individual animals as mentioned above. There was also a clinical tendency of reduction on other experimental time points but no statistical significance between the conjugate and control groups. The incidence of inner ear infection was also reduced by 51% in the conjugate group (11/39) relative to the control group (11/19) (p=0.029).

TABLE 14

Bacterial culture-positive rates (number of positives/total number) of MEFs from immunized chinchillas challenged with strain 9274

	Control		Conjugates (dLOS-TT, dLOS-HMP)		Total	
	right	left	right	left	control	conjugates
Day 3	5/5	3/5	6/10 (3/5[a], 3/5[b])	3/10 (1/5, 2/5)	8/10	9/20+
Day 7	5/5	4/5	8/10 (4/5, 4/5)	5/10 (3/5, 2/5)	9/10	13/20
Day 14	5/5	1/5	6/10 (3/5, 3/5)	2/10 (1/5, 1/5)	6/10	8/20

TABLE 14-continued

Bacterial culture-positive rates (number of positives/total number) of MEFs from immunized chinchillas challenged with strain 9274

	Control		Conjugates (dLOS-TT, dLOS-HMP)		Total	
	right	left	right	left	control	conjugates
Day 21	4/4	2/4	2/9* (0/5, 2/4)	0/9 + (0/5, 0/4)	6/8	2/18**
Total	19/19	10/19	22/39***	10/39*	29/38	32/78***

[a]number of animals having culture-positive MEFs/total number of animals for dLOS-TT-immunized animals;
[b]number of animals having culture-positive MEFs/total number of animals for dLOS-HMP-immunized animals.
Saline vs. conjugate-immunized group: +p = 0.07–0.08; *p < 0.05; **p < 0.01; ***p < 0.001.

[0099] When quantitative bacterial counts were performed on middle ear fluids recovered from infected animals at the right ear (challenged ear), there were significant differences between the conjugate and the control groups (**FIG. 4**) on each endpoint day. Conjugate-immunized animals had significantly lower middle ear fluid bacterial counts than did control animals (p=0.003).

[0100] Tympanic membrane observation of right (or challenge) ears by otoscopy showed signs of acute otitis media on day 1 postchallenge in the majority of animals. However, much more severe signs of infection were observed in the control group (average rating of 3.1±0.8) versus the dLOS-TT (1.1±1.2) or DLOS-HMP (1.8±1.4) groups (**FIG. 5**). By day 2, approximately 90% of control animals, but only 40% of conjugate-immunized animals, had a rating of 4. By day 4, all control animals showed severe signs of infection with a rating of 4 which remained up to day 21 (**FIG. 5**). In contrast, only about 55% of conjugate-immunized animals had a rating of 4 by day 4 and the maximum percentage of the highest rating at 4 was about 65% between days 5 and 11. By day 13, the conjugate-immunized animals showed apparent clearance of infection, with about 60% and 50% of dLOS-TT-immunized and DLOS-HMP-immunized, respectively, animals recovering from infection by day 21. The average numbers of TM observations were 4±0, 1.6±1.1, and 2±1.6 by day 21 (p<0.001), and 3.6±0.6, 2.5±1.9 and 2.8±1.6 at the whole period of 21 days (p<0.05) for saline, dLOS-TT and dLOS-HMP, respectively. Similar results were also obtained from left (or non-challenge) ears.

EXAMPLE 14

Immunogenicity of Conjugates in Chinchillas

[0101] Serum antibodies to NTHi 9274 LOS were determined by ELISA (Barenkamp, *Infect. Immun.*, 64:1246-1251, 1996; Gu et al., *Infect. Immun.*, 64:4047-4053, 1996). Briefly, 96-well plates were coated with 10 ig/ml LOS solution in PBS, pH 7.4 containing 0.1 mM $MgCl_2$ overnight at 4° C. The plates were washed and blocked with 5% fetal calf serum (FCS) in PBS for 1 hour, diluted sera were added and the mixtures were incubated for 2 hours. For IgG+IgM, rabbit anti-chinchilla IgG/IgM sera (1:500) (Barenkamp et al., supra) were added, and each mixture was incubated for 1 hour. For chinchilla IgG, protein A-alkaline phosphatase conjugate (Sigma) was added and the mixture was incubated for 1 hour. All steps were preformed at room temperature and PBS containing 0.05% Tween-20 was used in five washings between steps. Diluents for sera and conjugates were PBS containing 5% FCS and 0.05% Tween-20. Enzyme substrates were added for 30 minutes and the reactions were read with a microplate autoreaded (Bio-Tek Instruments Model EL311) at A_{405}. A chinchilla antisera raised against LOS was used as a positive control for each plate. Negative controls included buffer, alkaline phosphatase conjugate and serum alone. All negative controls gave optical density readings of less than 0.3. Chinchilla serum antibodies to TT or HMP were also measured by a similar ELISA to that described above, except TT or HMP was used as a coating antigen (5 μg/ml in 0.1 M Tris buffer, pH 9.8).

[0102] Three immunizations of saline did not elicit a rise of LOS antibodies in control chinchillas (Table 15). In contrast, both conjugates elicited significant levels of LOS antibodies with a 60- to 70-fold increase in IgG+IgM and a 20- to 40-fold increase in IgG after one injection. Increases of about 100-fold for IgG+IgM and 100- to 200-fold for IgG after three injections (P<0.001) were observed. IgG levels elicited by dLOS-TT were higher than those elicited by dLOS-HMP after three injections (p=0.0506). There was a negative correlation between LOS-IgG antibody levels and the bacterial counts of middle ear fluids among all 58 animals (r—0.298, P=0.023).

TABLE 15

Chinchilla antibody response to NTHi 9274 LOS elicited by conjugates

	Bleeding	GM (± SD range) ELISA titers for	
Immunogen	No.	IgG + IgM	IgG
Saline	1	33 (18–60)	11 (8–16)
	2	35 (20–61)	12 (8–18)
	3	36 (22–60)	13 (8–20)
	4	38 (24–60)	12 (8–18)
	5	38 (24–60)	12 (8–18)
dLOS-TT	1	30 (17–54)	16 (9–27)
	2	1950 (579–6566)	726 (88–5959)
	3	3771 (1430–9943)	1951 (403–9451)
	4	3984 (1730–9173)	3378 (1088–10932)
	5	3157 (1248–8902)	2865 (822–9989)
dLOS-HMP	1	34 (17–67)	13 (8–20)
	2	2431 (588–10044)	321 (40–2571)
	3	3643 (1069–12431)	764 (120–4853)
	4	2430 (567–10418)	1214 (174–8490)
	5	2292 (597–8792)	1286 (237–6978)

[a]58 chinchillas were immunized subcutaneously and intramuscularly with 3 doses of saline, dLOS-TT, or dLOS-HMP at 4-week intervals.
[b]blood samples were collected before (No. 1), 2 weeks after the 1st and 2nd immuniztion (No. 2 and 3), 10 days after the 3rd immunization (No.4), and before sacrifice (No. 5).
[c]saline-immunized sera or presera vs. conjugate-immunized sera: p < 0.001. There is no significant difference between dLOS-TT and dLOS-HMP except * vs. **:p = 0.0506.

[0103] Antibody responses to TT or HMP are shown in Table 16. Saline did not elicit TT or HMP antibodies. In contrast, both conjugates elicited significant levels of protein antibodies (IgG) with an approximate 300- to 1,400-fold increase after one injection and a 3,000- to 4,000-fold increase after three injections (p<0.001). Low levels of IgM were detected since the levels of IgG+IgM were similar to that of IgG only.

TABLE 16

Chinchilla antibody response to TT and HMP elicited by NTHi 9274 dLOS conjugates

Immunogen[a]	Bleeding No.[b]	GM (± range) ELISA Titers for: TT	HMP
Control	1	21(14~35)	17(14~44)
	2	22(17~29)	26(19~57)
	3	25(16~37)	22(14~51)
	4	24(19~41)	31(17~56)
	5	26(16~34)	24(19~SO)
dLOS-TT	1	26(18~37)	—[c]
	2	7,665(2,177~26,990)	—
	3	65,313(25,609~466,571)	—
	4	81,358(40,243~164,475)	—
	5	55,387(20,375~450,557)	—
dLOS-HMP	1	—	34(22~54)
	2	—	48,638(16,007~147,741)
	3	—	97,342(47,490~199,526)
	4	—	163,757(99,609~269,215)
	5	—	183,865(121,815~277,524)

[a], [b]: See Table 15 footnote.
[c]: not done

TABLE 17

Bactericidal activity of chinchilla antisera elicited by NTHi 9274 conjugates

Immunogen[a]	GM (± SD range) bactericidal titers[b] Presera	3rd immunized sera	Positive Rate (%)
Saline Control	<1:5	<1:5	0(0/19)[c]
dLOS-TT	<1:5	1:43(18~104)	45(9/20)
dLOS-HMP	<1:5	1:46(17~128)	53(10/19)
Conjugates[d]	<1:5	1:45(18~114)	49(19/39)

[a]See Table 15, footnote.
[b]Reciprocal of the highest dilution of sera showed >50% of killing to NTHi strain 9274.
[c]Saline vs. dLOS-TT or dLOS-HMP: $P < 0.001$.
[d]dLOS-TT group plus dLOS-HMP group.

EXAMPLE 15

Chinchilla Antibody Bactericidal Assay

[0104] Chinchilla pre- and postimniune sera (after three injections) were inactivated at 56° C. for 30 minutes and tested for bactericidal activity against NTHi 9274 by a microbactericidal assay (Gu et al., *Infect. Immun.*, 64:4047-4053, 1996). Briefly, a five-fold dilution of the initial sera, then two-fold serial dilutions were made in PBS containing calcium, magnesium and 0,1% gelatin (DPBSG), so that 50 μl of diluted sera was present in each well of a 96-well plate. Strain 9274 was grown on chocolate agar plates at 37° C. under a 5% CO_2 atmosphere overnight, and three to five colonies were transferred to another plate and incubated for 4-5 hours. The bacterial were diluted in DPBSG and 30 μl of bacterial suspension (about 3×10^3 CFU/ml) was added to each well. Infant rabbit serum (20 μl/well) was added as a source of complement (Pel-freez). The plates were incubated at 37° C. for 45 minutes. Subsequently, 50 μl of the mixture was transferred from each well onto chocolate agar plates. The plates were incubated at 37° C. under 5% CO_2 overnight and colonies were counted. Controls included complement, inactivated complement and a positive serum sample. The highest serum dilution caused >50% killing and was expressed as the reciprocal of bactericidal titer.

[0105] Neither presera not saline immunized sera showed bactericidal activity. However, 53% or 49% of DLOS-TT or dLOS-HMP immunized sera demonstrated a complement-mediated bactericidal activity against strain 9274 (Table 17). The titers ranged from 1:10 to 1:160. There was a positive correlation between LOS-IgG antibody levels (ELISA) and the bactericidal titers among all 58 animals.

[0106] Antibody levels are expressed as the geometric mean ELISA titers (reciprocal) of n independent observations ±standard deviation (SD). The MEF bacterial densities are expressed as the geometric CFU mean of n independent observations ±SD. The outcome of otoscopic observation is expressed as the mean of n independent observations ±SD at each time point. The significant differences of the above descriptions between the control and conjugate groups were tested with the student t test. The Fisher's exact test was employed to compare the proportion of infected animals between the control and conjugate groups.

EXAMPLE 16

Immunization of Humans with dLOS-TT Conjugate

[0107] Individuals are intramuscularly administered either 25 μg of the conjugate dLOS-TT or dLOS-HMP vaccine prepared as described in Examples 1-4 or a control vaccine. Boosters of 20 μg of conjugate or control vehicle are intramuscularly administered at 2, 4 and 15 months after the initial injection. Immunogenicity and bactericidal activity of the resulting antisera are determined in accordance with Examples 6 and 9. The frequency of occurrence of middle ear infections is then monitored over several years. The individuals receiving the conjugate vaccine have significantly fewer episodes of otitis media than the control subjects.

[0108] Multivalent vaccines comprising mixtures of dLOSs from different NTHi strains may also be used, particularly dLOSs from those strains which cause the most infections in humans.

[0109] It should be noted that the present invention is not limited to only those embodiments described in the Detailed Description. Any embodiment which retains the spirit of the present invention should be considered to be within its scope. However, the invention is only limited by the scope of the following claims.

What is claimed is:

1. A conjugate vaccine for nontypeable Haemophilus influenzae (NTHi), comprising lipooligosaccharide from which esterified fatty acids have been removed (dLOS), and an immunogenic carrier covalently linked thereto.

2. The vaccine of claim 1, wherein said immunogenic carrier is a protein.

3. The vaccine of claim 2, wherein said immunogenic carrier protein is selected from the group consisting of tetanus toxin/toxoid, NTHi high molecular weight protein, diphtheria toxin/toxoid, detoxified *P. aeruginosa* toxin A, cholera toxin/toxoid, pertussis toxin/toxoid, *Clostridium perfringens* exotoxins/toxoid, hepatitis B surface antigen, hepatitis B core antigen, rotavirus VP 7 protein, and respiratory syncytial virus F and G protein.

4. The vaccine of claim 3, wherein said immunogenic carrier protein is tetanus toxoid or NTHi high molecular weight protein.

5. A conjugate vaccine for nontypeable Haemophilus influenzae (NTHi), comprising lipooligosaccharide from which esterified fatty acids have been removed (dLOS), and an immunogenic carrier covalently linked thereto via a linker.

6. The vaccine of claim 5, wherein said linker is selected from the group consisting of adipic acid dihydrazide, ϵ-aminohexanoic acid, chlorohexanol dimethyl acetal, D-glucuronolactone and p-nitrophenylethyl amine.

7. The vaccine of claim 6, wherein said linker is adipic acid dihydrazide.

8. Isolated NTHi lipooligosaccharide detoxified by removal of ester-linked fatty acids therefrom.

9. A pharmaceutical composition comprising the vaccine conjugate of claim 1 in a pharmaceutically acceptable carrier.

10. The pharmaceutical composition of claim 9, further comprising an adjuvant.

11. The pharmaceutical composition of claim 10, wherein said adjuvant is alum.

12. A method of preventing otitis media caused by NTHi in a mammal, comprising administering to said mammal an effective immunoprotective amount of the conjugate vaccine of claim 1.

13. The method of claim 12, wherein said mammal is a human.

14. The method of claim 12, wherein said vaccine is administered by a route selected from the group consisting of intramuscular, subcutaneous, intraperitoneal, intraarterial, intravenous and intranasal.

15. The method of claim 14, wherein said administering step is intramuscular.

16. The method of claim 12, wherein said effective immunoprotective amount is between about 10 μg and about 50 μg.

17. The method of claim 12, further comprising injecting between about 10 μg and about 25 μg at about 2 and again at about 13 months after said administering step.

18. The method of claim 12, further comprising injecting between about 10 μg and about 25 μg at about 2, 4 and 16 months after said administering step.

19. A method of detoxifying lipooligosaccharide from NTHi, comprising removing ester-linked fatty acids therefrom.

20. The method of claim 19, wherein said ester-linked fatty acids are removed with hydrazine.

21. A method of making a conjugate vaccine against NTHi, comprising:

removing ester-linked fatty acids from NTHi lipooligosaccharide to produce DLOS; and

covalently binding said dLOS to an immunogenic carrier.

22. The method of claim 21, wherein said removing step comprises treatment with hydrazine.

23. The method of claim 21, further comprising the step of attaching said dLOS to a linker and attaching said linker to said carrier.

24. The method of claim 23, wherein said linker is selected from the group consisting of adipic acid dihydrazide, ϵ-aminohexanoic acid, chlorohexanol dimethyl acetal, D-glucuronolactone and p-nitrophenylethyl amine.

25. The method of claim 24, wherein said linker is adipic acid dihydrazide.

* * * * *

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10. The immunogenic composition of claim 7, wherein said polypeptide comprises the amino acid sequence Ser-Tyr-Gly-Arg-acetylated Lys-Lys-Arg-Arg-Gln-Arg-Cys (SEQ ID NO:03).

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11. The immunogenic composition of claim 7, wherein said polypeptide comprises the amino acid sequence Ser-His-Gly-Arg-acetylated Lys-Lys-Arg-Arg-Gln-Arg-Cys (SEQ ID NO:04).

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12. The immunogenic composition of claim 7, wherein said polypeptide is linked to a carrier.

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13. The immunogenic composition of claim 12, wherein said polypeptide is linked directly to the carrier.

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14. The immunogenic composition of claim 12, wherein said polypeptide is linked to said carrier through a linker.

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15. The immunogenic composition of claim 12, wherein said carrier is selected from a protein, a polysaccharide, a polyamino acid, an inactivated bacterial toxin, an inactivated bacterium, an inactivated viral particle, a lipid, and a liposome.

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16. The immunogenic composition of claim 12, wherein said carrier is selected from tetanus toxoid, diphtheria toxoid, purified protein derivative of *Mycobacterium tuberculosis*, and inactivated exotoxin A from *Pseudomonas aeruginosa*.

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17. The immunogenic composition of claim 7, further comprising an adjuvant.

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18. The immunogenic composition of claim 17, wherein the adjuvant is an aluminum salt adjuvant.

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19. A method of inducing an immune response to human immunodeficiency virus-1 (HIV-1) Tat protein in an individual, the method comprising administering an acetylated Tat

protein in an amount effective to induce an immune response to HIV Tat protein.

20. The method of claim 19, wherein said acetylated Tat protein is coupled to a carrier.

21. The method of claim 19, wherein said acetylated Tat protein is administered in a formulation comprising an adjuvant.

22. The method of claim 19, wherein said acetylated Tat protein is administered systemically.

23. The method of claim 22, wherein said acetylated Tat protein is administered subcutaneously.

24. A method of inhibiting transcriptional activation of human immunodeficiency virus (HIV), the method comprising administering an acetylated HIV Tat protein to the individual, wherein antibodies to the Tat protein are produced, and wherein said antibodies bind to serum Tat protein and reduce entry of the Tat protein into the cell.

25. An isolated antibody that specifically binds acetylated Tat protein.

26. The antibody of claim 25, wherein said antibody is a monoclonal antibody.

27. The antibody of claim 25, wherein said antibody is a polyclonal antibody.

28. The antibody of claim 25, wherein said antibody is a humanized mouse antibody.

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2001085649. PubMed ID: 11080476. **Acetylation** of **HIV**-1 **Tat** by CBP/P300 increases transcription of integrated **HIV**-1 genome and enhances binding to core histones. Deng L; de la Fuente C; Fu P; Wang L; Donnelly R; Wade J D; Lambert P; Li H; Lee C G; Kashanchi F. (Department of Biochemistry and Molecular Biology, UMDNJ-New Jersey Medical School, MSB E-635, Newark, New Jersey 07103, USA.) Virology, **(2000 Nov 25)** Vol. 277, No. 2, pp. 278-95. Journal code: 0110674. ISSN: 0042-6822. Pub. country: United States. Language: English.

AB The **HIV**-1 **Tat** protein is required for viral replication and is a potent stimulator of viral transcription. Although **Tat** has been extensively studied in various reductive paradigms, to date there is little information as to how this activator mediates transcription from natural nucleosomally packaged long terminal repeats. Here we show that CREB-binding protein (CBP)/p300 interacts with the **HIV**-1 **Tat** protein and serves as a coactivator of **Tat**-dependent **HIV**-1 gene expression on an integrated **HIV**-1 provirus. The site of **acetylation** of **Tat** was mapped to the double-lysine motif in a highly conserved region, (49)RKKRRQ(54), of the basic RNA-binding motif of **Tat**. Using HLM1 cells (**HIV**-1(+)/**Tat**(-)), which contain a single copy of full-length **HIV**-1 provirus with a triple termination codon at the first AUG of the **Tat** gene, we find that only wild type, and not K50A, K51A, or K50A/K51A alone or in combination of ectopic CBP/p300, is able to produce full-length infectious virions, as measured by p24 gag ELISAs. **Tat** binds CBP/p300 in the minimal histone acetyltransferase domain (1253-1710) and the binding is stable up to 0.85 M salt wash conditions. Interestingly, wild-type peptide 41-54, and not other **Tat** peptides, changes the conformation of the CBP/p300 such that it can acquire and bind better to basal factors such as TBP and TFIIB, indicating that **Tat** may influence the transcription machinery by helping CBP/p300 to recruit new partners into the transcription machinery. Finally, using biotinylated wild-type or **acetylated** peptides, we find that **acetylation** decreases **Tat**'s ability to bind the TAR RNA element, as well as to bind basal factors such as TBP, CBP, Core-Pol II, or cyclin T. However, the **acetylated Tat** peptide is able to bind to core histones on a nucleosome assembled **HIV**-1 proviral DNA.

Ac-Tat IC_{50} RKKRRQ

Isolated, acetylated Tat

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Citing References

2002325301. PubMed ID: 11956210. **Tat** acetyl-acceptor lysines are important for **human immunodeficiency virus** type-1 replication. Bres Vanessa; Kiernan Rosemary; Emiliani Stephane; Benkirane Monsef. (Institut de Genetique Humaine, CNRS UPR 1142, 141 rue de la Cardonille, 34396 Montpellier cedex 5, France.) The Journal of biological chemistry, **(2002 Jun 21)** Vol. 277, No. 25, pp. 22215-21. Electronic Publication: 2002-04-15. Journal code: 2985121R. ISSN: 0021-9258. Pub. country: United States. Language: English.

AB The **human immunodeficiency virus** type-1 trans-activator **Tat** is a transcription factor that activates the **HIV**-1 promoter through binding to the trans-activation-responsive region (TAR) localized at the 5'-end of all viral transcripts. We and others have recently shown that **Tat** is directly **acetylated** at lysine 28, within the activation domain, and lysine 50, in the TAR RNA binding domain, by **Tat**-associated histone acetyltransferases p300, p300/CBP-associating factor, and hGCN5. Here, we show that mutation of acetyl-acceptor lysines to arginine or glutamine affects virus replication. Interestingly, mutation of lysine 28 and lysine 50 differentially affected **Tat** trans-activation of integrated versus nonintegrated long terminal repeat. Our results highlight the importance of lysine 28 and lysine 50 of **Tat** in virus replication and **Tat**-mediated trans-activation.

Ac-Tat K^{28}, K^{50}

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2000012920. PubMed ID: 10545121. **HIV**-1 **tat** transcriptional activity is regulated by **acetylation**. Kiernan R E; Vanhulle C; Schiltz L; Adam E; Xiao H; Maudoux F; Calomme C; Burny A; Nakatani Y; Jeang K T; Benkirane M; Van Lint C. (Laboratoire de Virologie Moleculaire et Transfert de Gene, Institut de Genetique Humaine, UPR1142 Montpellier, 34396, France.) The EMBO journal, **(1999 Nov 1)** Vol. 18, No. 21, pp. 6106-18. Journal code: 8208664. ISSN: 0261-4189. Pub. country: ENGLAND: United Kingdom. Language: English.

AB The **human immunodeficiency virus** (**HIV**) trans- activator protein, **Tat**, stimulates transcription from the viral long-terminal repeats (LTR) through an RNA hairpin element, trans-activation responsive region (TAR). We and others have shown that trans-activator protein (**Tat**)-associated histone acetyltransferases (TAHs), p300 and p300/CBP-associating factor (PCAF), assist functionally in the activation of chromosomally integrated **HIV**-1 LTR. Here, we show that p300 and PCAF also directly **acetylate** **Tat**. We defined two sites of **acetylation** located in different functional domains of **Tat**. p300 **acetylated** Lys50 in the TAR RNA binding domain, while PCAF **acetylated** Lys28 in the activation domain of **Tat**. In support of a functional role for **acetylation** in vivo, histone deacetylase inhibitor (trichostatin A) synergized with **Tat** in transcriptional activation of the **HIV**-1 LTR. Synergism was TAR-dependent and required the intact presence of both Lys28 and Lys50. Mechanistically, **acetylation** at Lys28 by PCAF enhanced **Tat** binding to the **Tat**-associated kinase, CDK9/P-TEFb, while **acetylation** by p300 at Lys50 of **Tat** promoted the dissociation of **Tat** from TAR RNA that occurs during early transcription elongation. These data suggest that **acetylation** of **Tat** regulates two discrete and functionally critical steps in transcription, binding to an RNAP II CTD-kinase and release of **Tat** from TAR RNA.

Ac-Tat of IC^{50}

L31 ANSWER 3 OF 3 MEDLINE on STN

Citing References

2003355906. PubMed ID: 12887902. **Acetylation** of **Tat** defines a cyclinT1-independent step in **HIV** transactivation. Kaehlcke Katrin; Dorr Alexander; Hetzer-Egger Claudia; Kiermer Veronique; Henklein Peter; Schnoelzer Martina; Loret Erwann; Cole Philip A; Verdin Eric; Ott Melanie. (Deutsches Krebsforschungszentrum, D-69120 Heidelberg, Germany.) Molecular cell, (2003 Jul) Vol. 12, No. 1, pp. 167-76. Journal code: 9802571. ISSN: 1097-2765. Pub. country: United States. Language: English.

AB The **HIV** transcriptional activator **Tat** is **acetylated** by p300 at a single lysine residue in the TAR RNA binding domain. We have generated monoclonal and polyclonal **antibodies** specific for the **acetylated** form of **Tat** (AcTat). Microinjection of anti-AcTat **antibodies** inhibited **Tat**-mediated transactivation in cells. Similarly, the p300 inhibitor Lys-CoA and siRNA specific for p300 suppressed **Tat** transcriptional activity. Full-length synthetic AcTat bound to TAR RNA with the same affinity as unacetylated **Tat**, but formation of a **Tat**-TAR-CyclinT1 ternary complex was completely inhibited in the presence of AcTat. We propose that **Tat acetylation** may help in dissociating the **Tat** cofactor CyclinT1 from TAR RNA and serve to transfer **Tat** onto the elongating RNA polymerase II.

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Acetylation of Tat Defines a CyclinT1-Independent Step in HIV Transactivation

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Summary

The HIV transcriptional activator Tat is acetylated by p300 at a single lysine residue in the TAR RNA binding domain. We have generated monoclonal and polyclonal antibodies specific for the acetylated form of Tat (AcTat). Microinjection of anti-AcTat antibodies inhibited Tat-mediated transactivation in cells. Similarly, the p300 inhibitor Lys-CoA and siRNA specific for p300 suppressed Tat transcriptional activity. Full-length synthetic AcTat bound to TAR RNA with the same affinity as unacetylated Tat, but formation of a Tat-TAR-CyclinT1 ternary complex was completely inhibited in the presence of AcTat. We propose that Tat acetylation may help in dissociating the Tat cofactor CyclinT1 from TAR RNA and serve to transfer Tat onto the elongating RNA polymerase II.

Introduction

Transcriptional activity of the integrated HIV-1 provirus is regulated by the concerted action of cellular transcription factors and the viral transactivator Tat. In the absence of Tat, HIV transcription is highly inefficient because the assembled RNA polymerase II complex cannot elongate efficiently on the viral DNA template. Tat is a unique viral transactivator that binds to an RNA stem-loop structure called TAR, which forms at the 5′ extremity of all viral transcripts (reviewed in Garber and Jones [1999]). Tat binds to TAR via its C-terminal arginine-rich motif (ARM; amino acids 49–57) that is essential for RNA binding and nuclear localization. The N-terminal transactivation domain of Tat (amino acids 1–48) interacts directly with CyclinT1, a component of the positive-acting transcription elongation factor (P-TEFb) complex (Wei et al., 1998). CyclinT1 recruits the cyclin-dependent kinase 9 (CDK-9), the catalytic subunit of the separately identified "Tat-associated kinase" (TAK) (Herrmann and Rice, 1993). TAK/CDK-9 hyperphosphorylates the C-terminal domain (CTD) of the large subunit of the RNA polymerase II (RNAPII), leading to increased elongation efficiency of the polymerase complex (Zhu et al., 1997).

Tat activity also leads to the remodeling of a single nucleosome (nuc-1) positioned immediately downstream of the transcription start site (Verdin et al., 1993). Chromosomal integration, an essential step in the viral life cycle, leads to the packaging of the proviral DNA into an array of precisely positioned nucleosomes. These nucleosomes define two open regions of chromatin where transcription factors bind DNA (Verdin, 1991). Since Tat leads to nuc-1 remodeling, we proposed that Tat activates transcriptional elongation both by increasing the intrinsic ability of RNAPII to elongate efficiently and by remodeling the elongation block caused by nuc-1.

Chromatin can be remodeled either via the activity of multiprotein chromatin remodeling complexes or the activity of histone acetyltransferases (HATs) or via the combined activities of both (reviewed in Becker and Horz [2002]). Several transcriptional coactivators with intrinsic histone acetyltransferase activity interact with Tat, including p300/CBP (Benkirane et al., 1998; Hottiger and Nabel, 1998; Marzio et al., 1998), the Tat-interacting protein Tip60 (Kamine et al., 1996), and TAFII250 (Weissman et al., 1998). Histone acetyltransferases reversibly catalyze the transfer of acetyl groups on the ϵ-amino group of lysines in the N-terminal tails of histones H2A, H2B, H3, and H4, which form the core of nucleosomes. The level of acetylation of each lysine in the histone tail reflects the competing activities of HATs and histone deacetylases (HDACs) and plays a fundamental role in transcriptional regulation (Kornberg and Lorch, 1999).

We and others have reported that the HAT activity of p300 acetylates the Tat protein directly at a highly conserved lysine (K_{50}) in the ARM (Kiernan et al., 1999; Ott et al., 1999). We further demonstrated that acetylated Tat interacts with the bromodomain of the p300/CBP-associated factor (PCAF) (Mujtaba et al., 2002). A similar interaction has been previously described for acetylated histone H4 and the PCAF bromodomain (Dhalluin et al., 1999), establishing posttranslational histone modifications as a dynamic signaling platform for the recruitment of transcriptional regulators. The interaction between K_{50}-acetylated Tat and the PCAF bromodomain is required for Tat transactivation, since mutations of K_{50} in Tat or the acetyl-lysine binding site in PCAF suppress Tat transactivation (Dorr et al., 2002). Here, we have examined the role of Tat acetylation during HIV transactivation using modification-specific antibodies against acetylated Tat in nuclear microinjection experiments. Our observations support a model in which acetylation of K_{50} occurs as a critical step during HIV transcription

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that regulates cofactor binding to Tat, both by dissociation of CyclinT1 and association of modification-specific cofactors, such as PCAF.

Results

Tat Is Acetylated at K_{50} In Vivo

Modification-specific antisera have been raised against several proteins to detect unique posttranslational modifications. In particular, antisera specific for histone proteins containing unique acetylated lysines have proven extremely useful in assessing the role of acetylation in transcriptional regulation (Lin et al., 1989). Accordingly, we raised a specific antiserum against K_{50}-acetylated Tat by immunizing rabbits with a synthetic peptide corresponding to the ARM containing a single acetylated lysine at position 50 (rabbit anti-AcARM). The same peptide was injected into mice to generate monoclonal antibodies specific for K_{50}-acetylated Tat (anti-AcARM mAb). Monoclonal and polyclonal antibodies specifically recognized a synthetic Tat protein containing a single acetylated lysine at position 50 (AcTat) and showed no reactivity against synthetic unacetylated Tat (Figure 1A). The two antibodies also recognized Tat that had been acetylated by p300 in an in vitro acetylation reaction, confirming that p300 acetylates K_{50} in Tat (data not shown). Additional experiments showed no cross-reactivity with total cellular proteins, acetylated histone proteins, or unacetylated Tat at concentrations as high as 2 μg (data not shown).

The specificity was further tested in preincubation experiments. The antibodies were preincubated with ARM peptides or synthetic Tat proteins before Western blot analysis of AcTat. As expected, recognition by polyclonal and monoclonal antibodies was blocked with the acetylated ARM peptide (Figure 1B). The unacetylated ARM peptide had no effect, confirming the specificity of both antibodies for the acetylated immunogen (Figure 1B). Interestingly, only the polyclonal and not the monoclonal antibody was efficiently blocked by full-length acetylated Tat protein, indicating that both antibodies differed in their ability to recognize native, undenatured AcTat (Figure 1B). This result was confirmed by ELISA of synthetic Tat proteins coated on polystyrene plates. Again, only the rabbit anti-AcARM antibody recognized AcTat (Figure 1C).

Next, we analyzed cellular extracts with anti-AcARM antibodies. We generated a cell line that constitutively expressed a FLAG-tagged Tat protein from the HIV LTR by transducing Jurkat T cells with a Tat-encoding lentiviral vector (Jordan et al., 2001). Following treatment with trichostatin A (TSA), a specific inhibitor of cellular HDACs, AcTat was detected by Western blot analysis with anti-AcARM mAbs in cells expressing Tat, but not in control cells expressing GFP (Figure 1D). To enhance the signal, we performed immunoprecipitation with anti-FLAG monoclonal antibodies before Western blot analysis. Again, AcTat was detected in Tat-expressing, but not in GFP control cells (Figure 1E). Expression of AcTat was enhanced when cells were treated with TSA. However, immunodetection with anti-FLAG antibodies showed an overall upregulation of Tat expression by TSA, in agreement with the stimulatory effect of the drug on the HIV LTR (Van Lint et al., 1996).

To determine whether AcTat is associated with the HIV promoter in vivo, we performed chromatin immunoprecipitation (ChIP) assays in Jurkat cells expressing Tat or GFP from integrated HIV-based lentiviral constructs. Following formaldehyde cross-linking, solubilized chromatin was immunoprecipitated with polyclonal anti-AcARM antibodies. The DNA immunoprecipitated along with AcTat was analyzed by PCR with primers specific for the HIV LTR or for the cellular gene c-*fos*. In the presence of Tat, the HIV LTR sequence, and not the c-*fos* sequence, was enriched in the immunoprecipitated fraction, demonstrating that AcTat is specifically associated with the HIV LTR in vivo (Figure 1F). PCR analysis of the chromatin solution before immunoprecipitation (input) confirmed that the HIV and c-*fos* sequences were present in equivalent amounts in both samples.

Inhibition of Tat Activity by Anti-AcARM Immunoglobulins

Three different approaches were used to test the biological role of K_{50}-acetylated Tat in HIV transcription. First, we studied the effect of anti-AcARM antibodies on Tat transactivation after nuclear microinjection. HeLa cells stably expressing a full-length Tat protein (HeLa-Tat) or control HeLa cells were microinjected with an HIV LTR-luciferase construct as previously described (Dorr et al., 2002). A construct expressing enhanced GFP under the control of the CMV promoter (CMV-GFP) was coinjected to assess the number and viability of injected cells before harvest. Approximately 120 cells were microinjected per condition and luciferase values were on average 200-fold higher in HeLa-Tat cells than in control HeLa cells (Figure 2A).

Coinjection of purified rabbit anti-AcARM immunoglobulins into HeLa-Tat cells inhibited Tat transactivation (>85%), whereas preimmune immunoglobulins had no effect (Figure 2B). To validate this observation, a number of control experiments were performed (Figure 2B). (1) In agreement with their inability to recognize native AcTat, microinjected anti-AcARM mAbs had no effect on Tat transactivation. (2) Rabbit anti-AcARM immunoglobulins, immunodepleted with AcARM peptides, showed no inhibitory effect on Tat transcriptional activity. (3) An HIV LTR reporter, carrying a TAR mutation which prevents Tat binding, also showed no inhibitory effect of the antibodies. (4) No inhibitory effect was observed on Tat-independent promoters, such as the Rous sarcoma virus (RSV) LTR or the 5× UAS reporter activated by the Gal4-VP16 transactivator. These data demonstrate that the inhibition of Tat transcriptional activity by anti-AcARM antibodies is specific and that Tat acetylation at K_{50} is necessary for Tat transcriptional activity in vivo.

p300 Is a Critical Cofactor for Tat Transcriptional Activity

Second, we examined the effect of Lys-CoA, a recently described inhibitor of the p300 histone acetyltransferase activity, on Tat transactivation. When microinjected into nuclei of oocytes, Lys-CoA efficiently blocked p300-dependent transcription of MyoD RNA (Lau et al., 2000). Pilot experiments indicated that Lys-CoA could maxi-

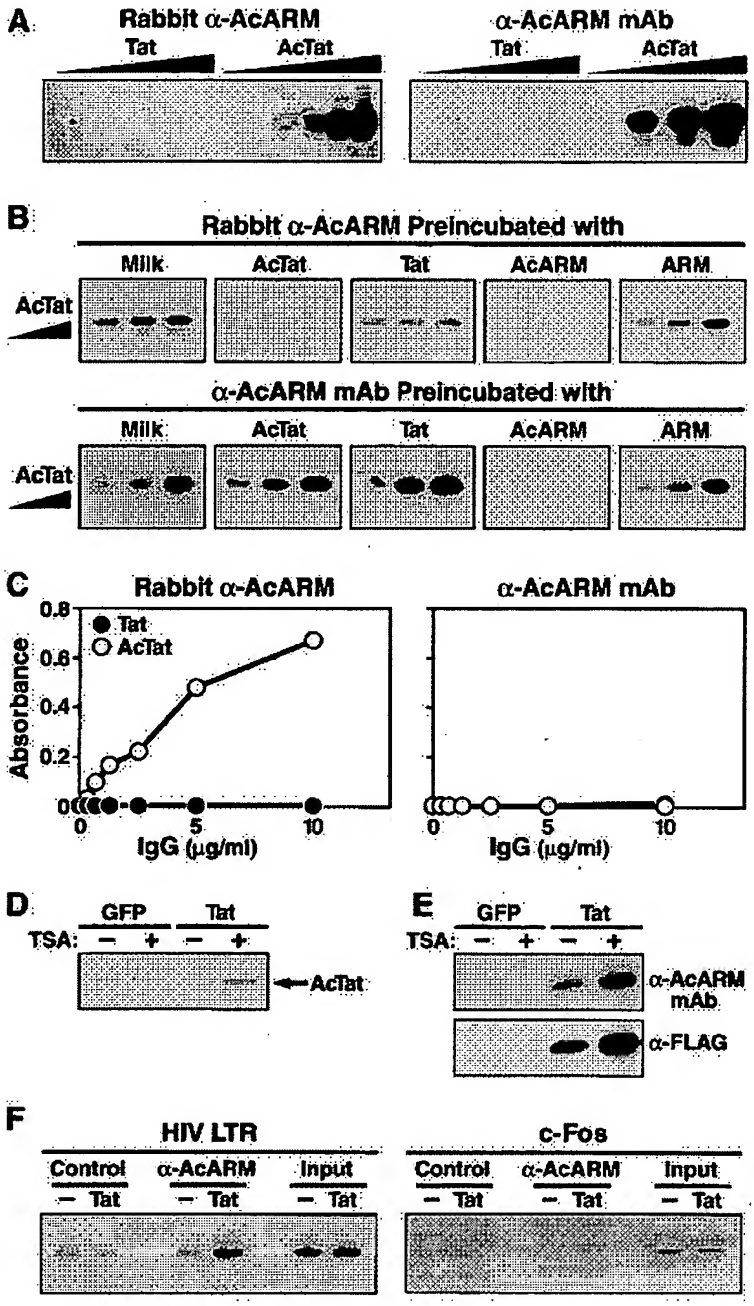

Figure 1. Generation of Monoclonal and Polyclonal Antibodies Specific for AcTat

(A) Western blot of synthetic Tat and AcTat using polyclonal or monoclonal anti-AcARM immunoglobulins (each 10 μg/ml).

(B) Western blot analysis of AcTat with polyclonal or monoclonal anti-AcARM immunoglobulins, preincubated with a 10× molar excess of the indicated agent.

(C) Tat (filled circles) or AcTat (open circles) was coated on ELISA plates (25 ng/well) and analyzed by ELISA at the indicated dilutions of polyclonal or monoclonal anti-AcARM immunoglobulins.

(D) Cellular lysates obtained from Jurkat T cell lines, expressing FLAG-tagged Tat or GFP, were analyzed by Western blotting with anti-AcARM monoclonal antibodies. Cells were treated with 400 nM trichostatin A (TSA) or DMSO for 16 hr.

(E) Cellular lysates were immunoprecipitated with anti-FLAG monoclonal antibodies before Western blotting with anti-AcARM or anti-FLAG monoclonal antibodies.

(F) Chromatin immunoprecipitation assay of Tat- or GFP-expressing Jurkat cells with polyclonal anti-AcARM antibodies and primers specific for the HIV LTR or for the c-*fos* gene as a control.

mally suppress Tat-mediated transactivation of the HIV promoter when injected as an 8 μM solution, which corresponds to an estimated final concentration of 0.8–1.6 μM in HeLa cell nuclei (data not shown). In three independent experiments, Lys-CoA inhibited Tat activity on the HIV LTR by 60%–70% (Figure 3A). Lys-CoA had no effect on the coinjected CMV-GFP reporter at the same concentration. Further, Lys-CoA had no effect on the LTR alone when coinjected into HeLa cells, indicating that the p300-HAT activity targets Tat directly.

In a third approach, we further demonstrated the critical role of p300 in Tat-mediated HIV transcription using short inhibitory RNA oligonucleotide duplexes (siRNA). siRNA specific for p300 (Gronroos et al., 2002) or GFP (Novina et al., 2002) were transfected into HeLa cells. Expression of p300 was monitored by Western blot analysis and confocal immunofluorescence microscopy. Three days after transfection, p300 expression was completely suppressed in cells transfected with siRNA specific for p300, but not for GFP (Figure 3B). Expression of the closely related CBP protein or of Lamin A/C was unaffected by p300-siRNA.

Next, we examined the effect of the p300-siRNA on Tat transactivation. Since CMV-GFP is included in the microinjection mix, we used another siRNA control specific for GL3 luciferase. It is important to note that the

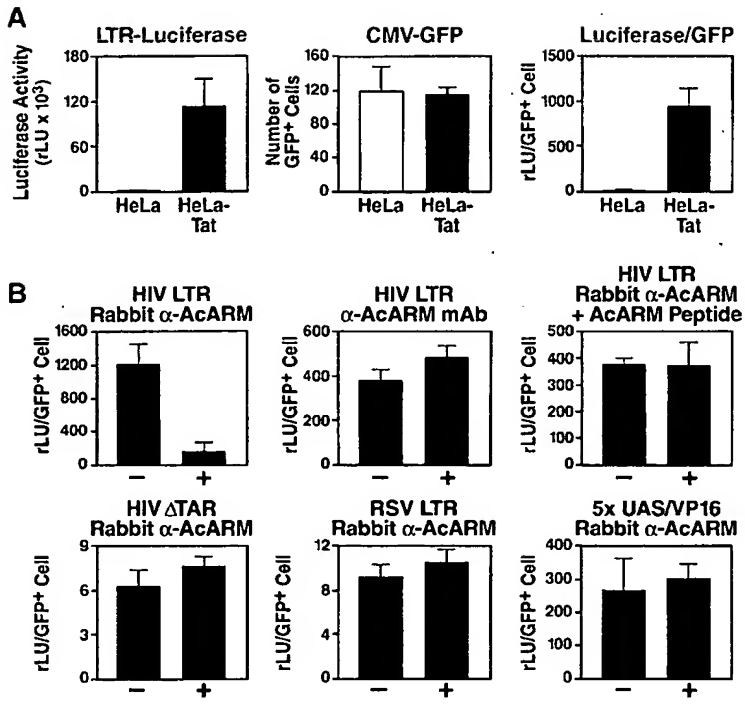

Figure 2. Inhibition of Tat Activity by Anti-AcARM Immunoglobulins

(A) Microinjection of an HIV LTR-luciferase construct into HeLa or HeLa-Tat cells, stably expressing full-length Tat. A GFP-expressing construct (CMV-GFP) was coinjected to count the number of injected cells before analysis of luciferase activity. The average (±SEM) luciferase values (relative light units, left), number of GFP-positive cells (middle), and luciferase values per GFP-positive cell (right) of three independent experiments are shown.

(B) Coinjection of anti-AcARM immunoglobulins (5 mg/ml) together with the HIV LTR-luciferase and CMV-GFP constructs into Hela-Tat cells (upper left and middle panels). Rabbit preimmune or monoclonal anti-p24 immunoglobulins were used as controls. Rabbit anti-AcARM and preimmune immunoglobulins were immunodepleted with the AcARM peptide before microinjection (upper right panel) or were coinjected with a TAR mutant LTR-luciferase construct (lower left panel), the Tat-independent RSV LTR-luciferase construct, or with the 5× UAS reporter transactivated by the Gal4-VP16 protein (lower middle and right panels). Average (±SEM) of three independent experiments is shown for each condition.

GL2 luciferase activity of the HIV LTR reporter used in this study is unaffected by GL3-siRNA (Elbashir et al., 2001). In microinjection experiments Tat transcriptional activity was suppressed 5-fold by p300-siRNA as compared to GL3-siRNA in HeLa cells expressing Tat (Figure 3C). No difference was observed in Tat-independent LTR activity when the TAR mutant luciferase construct was injected.

Similar results were obtained in HeLa cells microinjected with the wild-type LTR-luciferase construct and synthetic Tat (Figure 3C). To further demonstrate the critical role of p300 in Tat-mediated HIV transcription, viral infections with HIV-1-based vectors were performed. HeLa cells transfected with siRNA specific for either p300 or GL3 were infected with Tat-independent (LTR-GFP) or Tat-dependent lentiviral vectors (LTR-Tat-IRES-GFP), pseudotyped with the vesiculo stomatitis virus G protein (VSV-G). Viral expression was monitored by flow cytometric measurement of GFP. Again, Tat-mediated HIV transactivation, expressed as mean fluorescence intensity (MFI), was markedly suppressed, while basal HIV promoter activity was unaffected by the loss of p300 (Figure 3D). Cells transfected with p300-siRNA and GL3-siRNA were infected by the lentiviral vectors with the same efficiency at each infectious titer (as measured by the percentage of GFP+ cells; data not shown). This observation rules out that loss of p300 inhibited earlier steps in the viral life cycle before transcription.

AcTat Binds to TAR RNA, CyclinT1, and RNAPII, but Cannot Recruit CyclinT1 to TAR

The ARM of Tat is a highly conserved region that serves as the TAR RNA binding motif in Tat. The binding of Tat to the TAR hairpin is mediated by a 3 nucleotide U-rich bulge in the TAR stem while the loop sequences are contacted by the Tat cofactor CyclinT1. The interaction of Tat with CyclinT1 is thought to induce a conformational change in Tat that is important for ternary complex formation between Tat, TAR, and CyclinT1 (Wei et al., 1998).

To determine whether acetylation of the ARM alters binding to TAR RNA, we performed RNA gel mobility assays using the full-length synthetic Tat proteins. In the absence of CyclinT1, unacetylated Tat associated with wild-type TAR RNA with high affinity (Figure 4A). Binding was blocked when a bulge mutant RNA was used as a probe (Figure 4A). Acetylated Tat bound to TAR with affinities equal to that of unacetylated Tat; in fact, the binding affinity of AcTat appeared slightly enhanced, as formation of retarded bands occurred at one-third the concentration of unacetylated Tat (Figure 4B). As expected, mutations in the loop of TAR had little effect on the binding of Tat or AcTat (Figure 4B).

Addition of CyclinT1 to the reaction led to the formation of the ternary complex composed of Tat, TAR, and CyclinT1 (Figure 4B). Remarkably, unacetylated Tat and AcTat reacted differently in the presence of CyclinT1. While unacetylated Tat formed the expected Tat/TAR/CyclinT1 complex, AcTat could not. Addition of CyclinT1 did not alter the affinity of AcTat or unacetylated Tat for wild-type TAR RNA; however, as expected, ternary complex formation of unacetylated Tat with TAR and CyclinT1 was dependent on an intact TAR loop (Figure 4B). We also incubated the Tat/TAR/CyclinT1 complex with recombinant p300-HAT and acetyl-coenzyme A (AcCoA). Ternary complex formation, but not RNA binding of Tat, was suppressed when binding reactions were incubated in the presence, but not in the absence, of AcCoA, confirming these observations (data not shown).

Tat interacts with CyclinT1 through a domain that extends from amino acids 1–48 in Tat. This domain alone

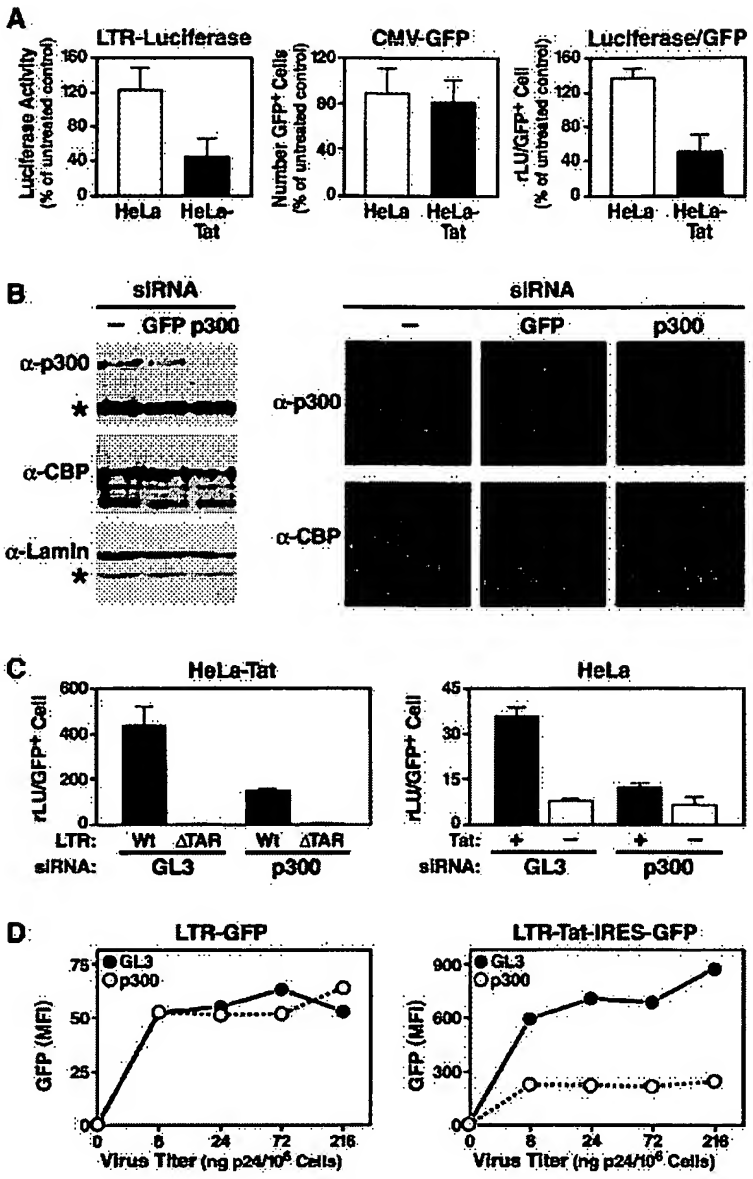

Figure 3. The Histone Acetyltransferase Activity of p300 Is Required for Tat Transcriptional Activity

(A) Microinjection of the p300-HAT inhibitor Lys-CoA (8 μM), HIV LTR-luciferase, and CMV-GFP into HeLa or HeLa-Tat cells. Each value is expressed as a percentage of the value obtained in control cells, injected with LTR-luciferase and CMV-GFP alone. Average (±SEM) luciferase activities (left), the corresponding number of GFP-positive cells (middle), and the ratios of luciferase values per GFP+ cell of three independent experiments are shown.

(B) Left, Western blot analysis of nuclear extracts prepared from HeLa cells transfected with siRNA specific for p300 or GFP. The asterix in the upper panel marks an unspecific band at 150 kDa, in the lower panel a cleavage product of lamin A/C. Right, confocal immunofluorescence microscopy of siRNA-transfected HeLa cells, visualized with Cy3-labeled secondary antibodies.

(C) Microinjection experiments with HIV LTR-luciferase and CMV-GFP constructs in HeLa-Tat or HeLa cells, transfected with siRNAs specific for p300 or GL3 luciferase. HeLa cells were injected in the absence or presence of Tat (30 μg/ml).

(D) HeLa cells, transfected with siRNAs specific for GL3 (filled circles) and p300 (open circles), were infected with GFP- or Tat and GFP-expressing lentiviral vectors. Transcriptional activity of the HIV LTR is measured as mean fluorescence intensity (MFI) of GFP.

is sufficient to bind to CyclinT1 and to compete with full-length Tat to prevent assembly of the Tat/CyclinT1/TAR complex in vitro (Wei et al., 1998). To test whether in the absence of TAR AcTat binds CyclinT1, we performed GST pulldown experiments with synthetic Tat proteins and GST-CyclinT1 (Figure 4C). Tat and AcTat bound to wild-type GST-CyclinT1 with equal affinities, indicating that the transactivation domain in each synthetic protein was intact. Neither Tat nor AcTat bound to a mutant CyclinT1 in which cysteine at position 261 had been replaced with an alanine or to GST alone, confirming the specific binding conditions.

Evidence has been presented that the formation of the Tat/CyclinT1/CDK9 complex at TAR RNA represents an early step during HIV transcription elongation. During late stages, the Tat protein associates directly with the elongating RNAPII, independently of TAR RNA and CyclinT1 (Cujec et al., 1997; Mavankal et al., 1996). Direct interaction with RNAPII required an intact ARM of Tat (Mavankal et al., 1996; Zhou and Rana, 2002) and occurred after recruitment of Tat to TAR (Keen et al., 1997). Next, we compared the ability of both acetylated and nonacetylated Tat to interact with RNAPII. Biotinylated Tat or AcTat were incubated with nuclear extracts and the bound proteins analyzed by Western blotting. This experiment demonstrated that AcTat interacted with the hyperphosphorylated RNAPII complex with higher affinity than Tat (Figure 4D). In the same experiment, no binding of Tat or AcTat to the nonphosphorylated RNAPII was observed. These data indicate that while AcTat is unable to bind with CyclinT1 to TAR, it shows increased affinity for the elongation-competent RNAPII.

Discussion

Tat is thought to serve as a bridge to recruit CyclinT1/CDK9 as part of the P-TEFb complex to the HIV promoter, thereby facilitating transcriptional elongation.

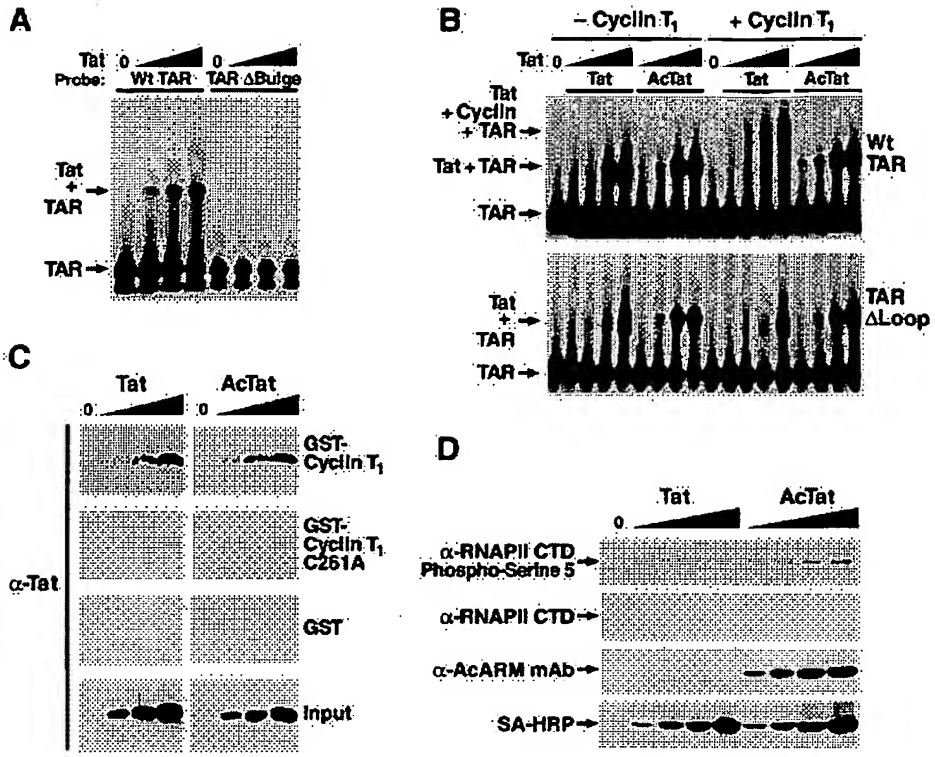

Figure 4. Acetylation of Tat Blocks the Formation of the Ternary Tat/TAR/CyclinT1 Complex, but Allows Binding to RNAPII

(A) Bulge-dependent binding of Tat to TAR. Radiolabeled riboprobes corresponding to the HIV TAR element or a mutated form (TARΔBulge) were incubated with 0, 40, 80, and 160 ng of Tat and analyzed on a 4% Tris-glycine gel.

(B) High-affinity binding of AcTat to TAR RNA, but not to CyclinT1. The wild-type TAR probe was incubated with synthetic Tat or AcTat (0, 1.6, 8, 40, and 200 ng) in the absence or presence of recombinant CyclinT1. The same experiment with a mutated TAR RNA probe containing mutations in the apical loop is shown below (TARΔLoop).

(C) Binding of AcTat to CyclinT1 in the absence of TAR. Increasing amounts (0, 0.5, 1, and 2 μg) of Tat or AcTat were incubated with GST-CyclinT1, Tat binding-deficient GST-CyclinT1 (C261A), or GST alone. Bound proteins were analyzed with monoclonal anti-Tat antibodies, directed against the N terminus of Tat. 50% of input Tat proteins are shown.

(D) Binding of Tat and AcTat to RNAPII. Biotinylated Tat or AcTat proteins (0, 1, 2, 4, and 8 μg) were incubated with nuclear extracts, precipitated with streptavidin-sepharose, and analyzed with modification-specific antibodies directed against the CTD of RNAPII. AcTat was detected with monoclonal anti-AcARM antibodies. Both Tat proteins reacted with horseradish peroxidase-conjugated streptavidin (SA-HRP).

The cooperative binding of Tat with CyclinT1 to TAR RNA leads to the recruitment of CDK9 to the transcription initiation site. Binding of CDK9 close to this site leads to the hyperphosphorylation of the CTD of RNAPII and to an increase in polymerase processivity. While this model has been validated extensively both genetically and biochemically, the identification of a new post-translational modification of Tat by acetylation brings an added level of complexity to this paradigm.

Three independent observations reported in this manuscript support the model that acetylation of Tat is necessary for its transcriptional activation of the HIV promoter. First, we show that nuclear microinjection of an antiserum specific for AcTat suppressed Tat-mediated transactivation. Second, we observed that Lys-CoA, a specific inhibitor of the acetyltransferase activity of p300/CBP, inhibited Tat transactivation. Previous experiments have documented that p300/CBP directly acetylated Tat in vitro and interacted with Tat to activate the HIV promoter. This synergy was dependent on the HAT domain of p300 (Kiernan et al., 1999) and on the conservation of the Tat acetylation site (K_{50}) (Ott et al., 1999). Interestingly, we have also observed that direct microinjection of AcTat can reverse the suppressive effect of Lys-CoA, while the unacetylated Tat protein had no effect (data not shown). These findings collectively support the hypothesis that acetylation of Tat by p300/CBP represents a necessary step in the transactivation process.

Third, we used RNA-mediated interference (RNAi) to suppress p300 expression. Interestingly, suppression of p300, and not of CBP, led to a 5-fold reduction of Tat activity in microinjection and viral infection experiments. Whether the residual Tat transcriptional activity observed after p300 knockdown is due to the presence of CBP or whether p300 and CBP differ in their roles as Tat acetyltransferases in vivo, remains to be determined. Although functionally highly overlapping, distinct properties of p300 and CBP in cell development and signaling responses have been identified (reviewed in Vo and Goodman [2001]). Residual Tat activity was also observed after treatment with Lys-CoA, which targets p300 and CBP HAT activity (Lu et al., 2002; Victor et al., 2002). This incomplete suppression might be due to the subop-

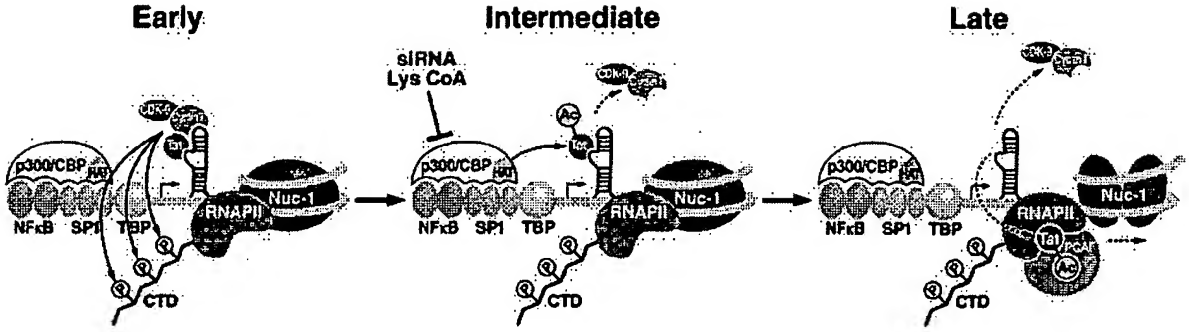

Figure 5. Tat Acetylation Serves as a Molecular Switch between Early and Late Steps of HIV Transcription Elongation
See Discussion for details.

timal dose of Lys-CoA used in this study or might represent the remaining activity of a K_{50} acetyltransferase different from p300 and CBP, i.e., human GCN5 (Col et al., 2001) or PCAF (our unpublished data).

The acetylation of the Tat ARM could potentially affect any of the biological activities associated with this domain, including RNA binding, nuclear/nucleolar import, and protein stability. The ARM domain of Tat mediates RNA binding via the bulge region of TAR. While a small peptide corresponding to the ARM domain alone or the purified HIV Tat protein can bind to the TAR RNA alone in vitro, the relevance of this binding reaction to transactivation in vivo is not clear. In fact, extensive biochemical and genetic evidence suggest that the recognition of TAR by the ARM domain of Tat is not sufficient for binding of Tat to TAR in vivo and that the transactivation domain of Tat is necessary both for binding to TAR and for transactivation (reviewed in Cullen [1998]). The cellular cofactor that binds the transactivation domain of Tat and promotes specific Tat binding to TAR is CyclinT1 (Wei et al., 1998). The Tat/CyclinT1 complex binds to the TAR hairpin both via Tat and the TAR bulge and via CyclinT1 and the TAR loop.

Our observation that acetylated Tat was unable to form a ternary complex with CyclinT1 on TAR therefore suggests that Tat acetylation, by preventing CyclinT1 recruitment, leads to a dissociation of Tat from TAR in vivo. Interestingly, our experiments also indicate that Tat acetylation did not modify its ability to bind to CyclinT1 independently of TAR. In contrast to previous reports, we observed no effect of Tat acetylation on its binding to TAR independently of CyclinT1 (Kiernan et al., 1999). As discussed above, the biological relevance of this observation is unclear since Tat does not appear to be able to use TAR in vivo in the absence of CyclinT1.

It is not clear at this point how Tat acetylation prevents the formation of a ternary complex with TAR and CyclinT1 while not affecting the binding of Tat to CyclinT1. In the absence of TAR, amino acids 1–48 in Tat are sufficient to interact with CyclinT1 (Wei et al., 1998). A recently solved Tat structure showed K_{50}, the site of Tat acetylation, to be exposed at the surface of Tat, between the RNA binding motif of Tat and its CyclinT1 interaction domain, the cysteine-rich region. Insertion of an acetyl group in this region is likely to disturb the protein surface responsible for the binding of Tat/Cyclin T to TAR (Peloponese et al., 2000). In addition, RNA-protein cross-linking experiments showed that K_{50} of Tat represents a key residue at the interface between the Tat transactivation domain, the TAR loop and CyclinT1 (Richter et al., 2002).

Importantly, we have also observed that K_{50} can be acetylated by p300 in the context of the ternary complex Tat/TAR/CyclinT1. This observation suggests that Tat could become acetylated at the level of the HIV promoter when it comes into close contact with p300, interacting with Tat directly or with several transcription factors bound at the level of the HIV promoter, including NF-κB, Sp1, and NFAT (Billon et al., 1999; García-Rodríguez and Rao, 1998; Perkins et al., 1997). These observations are consistent with a role of Tat acetylation as a molecular signal leading to the disruption of the Tat/CyclinT1/TAR complex. Dissociation of this complex could play an important role in the "recycling" of Tat from one transcript to the next transcript. In contrast to a classical transcription factor, which is stably bound at the level of DNA and mediates the successive recruitment of RNAPII transcription complexes, Tat is bound to the primary transcript. This fact implies that the Tat protein will be consumed during successive transcription cycles. Tat acetylation could therefore serve as a switch to release Tat from the primary transcript to a pool of Tat proteins available for new transcription cycles.

Based on the observation that AcTat binds to the elongation-competent RNAPII complex with higher affinity than Tat, we favor an alternative model where Tat acetylation serves as a molecular switch between the early TAR-dependent and late TAR-independent phases of HIV transcriptional elongation (Figure 5). According to this model, the first stage is mediated by unacetylated Tat acting together with CyclinT1 through TAR RNA to recruit the P-TEFb complex to the HIV promoter. When bound to TAR, Tat is subjected to acetylation by p300 bound at the level of the HIV promoter. Acetylation of Tat by p300 leads to the disruption of the Tat/CyclinT1/TAR complex and to the high-affinity recruitment of Ac-Tat to the elongating RNAPII.

We have recently reported that the acetylated lysine in Tat serves as a novel protein/protein interface leading to the recruitment of the PCAF transcriptional coactivator via its bromodomain (Dorr et al., 2002; Mujtaba et al., 2002). Interestingly, in other systems, PCAF has been

shown to be specifically associated with the elongating polymerase complex while p300 was found bound at the transcriptional start site (Cho et al., 1998). Since Tat activity in vivo is associated with the remodeling of a single nucleosome called nuc-1 at the transcriptional start site, we speculate that AcTat bound to the elongating RNAPII leads to the recruitment of a chromatin remodeling complex, including the HAT activity of PCAF. Future experiments will further define the role of AcTat with respect to the elongation competence of the RNAPII complex and the chromatin organization of the integrated HIV promoter.

Experimental Procedures

Preparation and Use of Monoclonal and Polyclonal α-AcARM Antibodies

Chemically synthesized K_{50}-acetylated ARM peptides were conjugated to keyhole limpet hemocyanin (Pierce, Rockford, IL), mixed with complete Freund's adjuvant (Sigma, St. Louis, MO), and injected into rabbits. Four boosts were performed with incomplete Freund's adjuvant in rabbits. Monoclonal antibodies (clone TALT5.24) were generated with the same immunogen according to a rapid repetitive immunization protocol (Bynum et al., 1999). Immunoglobulin G were purified on Gammabind Plus Sepharose (Amersham Pharmacia Biotech, Uppsala, Sweden). For ELISA, polystyrene plates were coated overnight at 4°C with 100 ng of Tat or AcTat protein per milliliter carbonate buffer (500 mM $NaHCO_3$). Plates were incubated with IgGs in milk and developed with horseradish peroxidase-conjugated secondary antibodies (Jackson ImmunoResearch Laboratories) and 5 mg/ml o-phenylenediamine (Sigma). Immunoprecipitations of the FLAG-Tat protein were performed with 10 μg/ml M2 monoclonal antibody (Sigma) as previously described (Ott et al., 1999). For Western blotting with polyclonal α-AcARM IgGs, 0, 0.3, 1, 3, 10, 30, and 100 ng of synthetic Tat proteins were loaded; for analysis with monoclonal α-AcARM IgGs, 0, 0.5, 1, and 2 μg of each protein were loaded. AcTat concentrations in blocking experiments were 125, 250, and 500 ng/lane for the monoclonal, and 31, 62, and 125 ng/lane for the polyclonal antibody.

ChIP Assays

Both GFP- and Tat-transduced Jurkat cells were fixed for 10 min with 1% formaldehyde. Soluble chromatin was prepared and purified by cesium chloride isopicnic centrifugation as previously described (Orlando et al., 1997). Precleared chromatin solutions were incubated overnight at 4°C with 5 μl of the antibody (rabbit α-AcARM IgGs). Immune complexes were collected with protein A-agarose preblocked with sonicated salmon sperm DNA (Upstate Biotechnology, Waltham, MA). Formaldehyde cross-links were reverted by incubating the samples at 65°C overnight in the presence of 200 mM NaCl. One-tenth of the immunoprecipitated DNA was used in PCR reactions using the following primer pair for HIV LTR detection (5′-TTGCCTGTACTGGGTCTCTCTG-3′ and 5′-TCGCTTTCAGGTC CCTGTTCG-3′) and the following pair for *c-fos* detection (5′-TGC ATAGAAGGACCCAGATAGGTC-3′ and 5′-TCAATGACCCTGAGCC CAAG-3′).

Synthetic Tat Proteins

Solid-phase peptide synthesis of 72 amino acid Tat was performed with a sequence derived from the isolate HIV-1_{BRU} by standard Fmoc-Strategy as previously described (Dorr et al., 2002). For biotinylated proteins, biotinylation was carried out at the last step of the synthesis. All proteins were fully deprotected with trifluoracetic acid, containing 3% triisopropylsilane and 5% water. The peptides were purified to homogeneity by reverse-phase high-pressure liquid chromatography (Shimadzu Scientific Instruments Inc., Columbia, MD). The correct molecular masses for the peptides were established by positive-ion ESI mass spectra recorded on an ion trap Finnigan LCQ mass spectrometer (Bremen, Germany).

Cells and Plasmids

HeLa-Tat cells were provided by Peter Krammer (DKFZ, Heidelberg, Germany). Jurkat-GFP cells and the lentiviral vectors to generate Jurkat-Tat cells are described in Jordan et al., (2001). The HIV LTR-luciferase construct was described before (Emiliani et al., 1998). The TAR bulge mutation was generated by complete deletion of the 3 nucleotide bulge introduced via QuickChange site-directed mutagenesis (Stratagene, La Jolla, CA). The RSV LTR-luciferase construct was a gift of Heike Pöpperl (DKFZ), and the CMV-GFP construct was obtained from Clontech (Palo Alto, CA). The 5× UAS construct, in which 5× Gal4 binding sites were cloned upstream of the TK promoter (Puigserver et al., 1999), was provided by Bruce Spiegelman (Harvard Medical School, Boston, MA). GST-CyclinT1 and mutant GST-CyclinT1 plasmids were provided by Katherine Jones (Salk Institute, San Diego, CA). The lentiviral vectors LTR-GFP and LTR-Tat-IRES-GFP vectors and the method to generate viral particles pseudotyped with VSV-G are described in (Jordan et al., 2001). Both vectors are minimal nonreplicative HIV-1 genomes flanked by two LTRs containing viral *cis*-acting sequences necessary for packaging and infection (Dull et al., 1998). Viral particles were quantified with an HIV-1 p24 ELISA assay (NEN Life Science Products Inc., Boston, MA).

Microinjection Experiments

Microinjections were performed at room temperature with a Zeiss automated injection system (Carl Zeiss, Goettingen, Germany). Samples were prepared as a 20 μl injection mix containing the luciferase reporter constructs (100 μg/ml) and CMV-GFP (50 μg/ml) constructs in sterile water or phosphate buffer. In individual experiments, α-AcARM or preimmune immunoglobulins (5 mg/ml), Lys-CoA (8 μM), or synthetic Tat (30 μg/ml) were included in the mix. Four hours after injection, cells were examined on a Zeiss Axiovert microscope to determine the number of GFP-positive cells, washed in cold phosphate buffer, and stored at −70°C for luciferase assays (Promega, Madison, WI). The α-p24 monoclonal antibody (183-H12-5C) was used in control microinjections with α-AcARM mAbs and was obtained through the AIDS Research and Reference Reagent Program, NIH (Chesebro et al., 1992). For immunodepletion of rabbit α-AcARM or preimmune antibodies, immunoglobulins were incubated with biotinylated AcARM peptides prebound to streptavidin-sepharose beads (30 min at room temperature), centrifuged, and supernatant coinjected into HeLa-Tat cells.

siRNA Transfections

Transient transfections of siRNAs (Dharmacon Research, Lafayette, CO) were performed using oligofectamine (Invitrogen, Carlsbad, CA). 72 hr after transfection, nuclear extracts were prepared according to a rapid protocol (Osborn et al., 1989) or cells were fixed with paraformaldehyde for immunofluorescence microscopy with Cy3-conjugated α-rabbit secondary antibodies (Jackson Immunoresearch Laboratories, West Grove, PA). Antibodies α-p300 (N-15) and α-CBP (A-22, both Santa Cruz Biotechnology, Santa Cruz, CA) and α-Lamin A/C polyclonal antibodies (Cell Signaling Technology Inc., Beverly, MA) were used according to the manufacturers' recommendations. Confocal images were acquired with an Olympus BX60 microscope equipped with a Radiance 2000 confocal setup (Bio-Rad, Hercules, CA).

RNA Gel Shift Experiments

TAR RNAs were synthesized from HindIII-linearized pGEM3Zf-TAR constructs, which contained an oligonucleotide corresponding to the HIV_{LAI} TAR sequence (nucleotides 17–44) ligated into the EcoR1-HindIII cloning sites, downstream of the T7 RNA polymerase start site. In the TARΔbulge construct, thymidine 23 was replaced with an adenosine, and the TARΔloop construct contained C30→A, T31→G, and G33→T mutations. In vitro transcription reactions were performed with the Riboprobe system (Promega) with 1 μg of linearized plasmids and 50 μCi of ^{32}P-CTP (Amersham Pharmacia Biotech). Transcripts were treated with 2 U DNase I (Promega), extracted with a phenol:chloroform mixture, and purified over a Nick column (Amersham Pharmacia Biotech). Gel mobility reactions (final volume, 16 μl) were carried out at 30°C as described (Wei et al., 1998) with 2×10^4 cpm TAR probes/reaction, indicated concentrations of Tat

proteins, and 160 ng of GST-cleaved CyclinT1 (amino acids 1–303). RNA binding complexes were separated on a prerun 4% Tris-glycine gel.

GST Binding Assays

Full-length GST-CyclinT1, GST-CyclinT1C261A, or GST alone were expressed in the BL21 strain of *Escherichia coli* and purified on glutathione-Sepharose beads (Amersham Pharmacia Biotech) as described (Herrmann and Rice, 1993). For binding assays, 1 μg of bead-coupled GST-CyclinT1, GST-CyclinT1C261A, or GST alone was incubated with Tat and AcTat in 100 μl modified buffer C for 3.5 hr at 4°C. Buffer C is described in Fujinaga et al. (1999) and was used without $ZnCl_2$ and SDS. Beads were washed three times with modified buffer C containing 1 M KCl, boiled in Laemmli buffer, and supernatant was analyzed by immunoblotting using monoclonal anti-Tat antibodies (clone AC11.AE12; Covance Research Products, Cumberland, VA).

RNA Polymerase II Binding

Synthetic biotinylated nonacetylated and acetylated Tat proteins were incubated for 2 hr with 250 μg 293 nuclear extracts prepared according to Dignam et al. (1983) in buffer conditions described in Mavankal et al. (1996). Complexes were collected with streptavidin-sepharose beads, separated by SDS-PAGE electrophoresis and analyzed with α-RNAPII antibodies (clones H14 and 8WG16; Covance Research Products) or with α-AcARM mAbs and HRP-conjugated Streptavidin (Jackson Immunoresearch Laboratories).

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Tat Acetyl-acceptor Lysines Are Important for Human Immunodeficiency Virus Type-1 Replication*

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The human immunodeficiency virus type-1 *trans*-activator Tat is a transcription factor that activates the HIV-1 promoter through binding to the *trans*-activation-responsive region (TAR) localized at the 5′-end of all viral transcripts. We and others have recently shown that Tat is directly acetylated at lysine 28, within the activation domain, and lysine 50, in the TAR RNA binding domain, by Tat-associated histone acetyltransferases p300, p300/CBP-associating factor, and hGCN5. Here, we show that mutation of acetyl-acceptor lysines to arginine or glutamine affects virus replication. Interestingly, mutation of lysine 28 and lysine 50 differentially affected Tat *trans*-activation of integrated *versus* nonintegrated long terminal repeat. Our results highlight the importance of lysine 28 and lysine 50 of Tat in virus replication and Tat-mediated *trans*-activation.

Human immunodeficiency virus type-1 (HIV-1)[1] Tat is a regulatory protein encoded by two exons localized on either side of the *env* gene. Tat is a multifunctional protein, absolutely required for virus replication and AIDS progression. Besides its primary function as the viral transcription factor, Tat has been proposed to be required for efficient reverse transcription (1). Tat is secreted from infected cells (2, 3), whereupon it binds to neighboring cells through electrostatic interactions, chemokine receptors (4), or cell surface integrins (5). Extracellular Tat is a cellular toxin that increases the efficiency of virus dissemination and reduces antiviral immunity to promote HIV-1 disease (6). Cells treated with Tat show increased expression of chemokine receptors (7, 8), decreased proliferation (9, 10), and apoptosis of bystander cells (11, 12). Furthermore, Tat has been shown to have chemokine-like properties that may serve to recruit chemokine receptor-expressing monocytes/macrophages toward HIV-producing cells and facilitate infection (3–5). Finally, extracellular HIV-1 Tat protein has been shown to selectively inhibit the entry and replication of T-cell tropic X4, but not macrophage-tropic R5, virus in peripheral blood mononuclear cells, which has been proposed as a mechanism to select against X4 viruses, thereby influencing the early course of HIV-1 disease (13).

The primary function attributed to Tat is its role in HIV-1 promoter activation. Tat is an atypical transcriptional activator that functions through binding, not to DNA, but to a short leader RNA, *trans*-activation responsive region (TAR) localized at the 5′ termini of all viral transcripts (14–16). Interaction between Tat and TAR is necessary for HIV-1 transcription both *in vivo* (17, 18) and *in vitro* (19, 20). Tat transcriptional activity on the HIV-1 promoter is tightly regulated by cellular factors (reviewed in Ref. 21): Tat-associated-kinases (22–24) and Tat-associated histone acetyltransferases (25–28). Tat-associated kinase was identified as the kinase subunit of the positive transcription elongation factor b (29–33). Positive transcription elongation factor b is composed of a regulatory subunit, cyclin T1, and a catalytic subunit, CDK9, which phosphorylates the carboxyl-terminal domain of the large subunit of RNA polymerase II (32, 34). Hyperphosphorylation of the RNA polymerase II carboxyl-terminal domain leads to productive elongation of transcription (24, 32, 33, 35–37). Tat interacts with cyclin T1 to recruit positive transcription elongation factor b to the HIV-1 TAR element and to stimulate elongation of transcripts originating from the viral long terminal repeat (LTR) (32, 33). The other class of Tat co-activators, Tat-associated histone acetyltransferases, are composed of p300/CBP, p300/CBP-associating factor (PCAF) (25–27), and hGCN5 (28). Tat-associated histone acetyltransferases induce the activation of chromatinized HIV-1 LTRs (26, 27), presumably through acetylation of histones. Tat may also use the cellular acetylation pathway to control the expression of various cellular genes (38, 39). We and others have recently shown that Tat-associated histone acetyltransferases also directly acetylate the Tat protein in two different domains. Whereas p300 and hGCN5 acetylate lysine 50 within the RNA binding domain (28, 40–42), PCAF acetylates lysine 28 in the activation domain (40). Thus, this novel post-translational modification of Tat was found to govern two essential interactions necessary for HIV-1 transcription: binding of Tat to TAR and to positive transcription elongation factor b.

In the present work, we analyzed the role of Tat acetyl-acceptor lysines in virus replication. We show that mutation of lysine 28 and lysine 50 to either arginine or glutamine severely affected the replication of HIV-1 in a T-cell line. Additionally, the effect of Lys^{28} and Lys^{50} mutation on Tat *trans*-activation was dependent on the promoter context (integrated *versus* nonintegrated LTR). Our results highlight the importance of lysine 28 and lysine 50 of Tat in virus replication and the mechanism of Tat-mediated *trans*-activation.

EXPERIMENTAL PROCEDURES

Plasmid Constructs—pLTR-luc wild-type has been described (40). A FLAG sequence was introduced in the COOH terminus of pTat wild type, which was used as a template for mutagenesis. pTat-K28R, pTat-

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[1] The abbreviations used are: HIV-1, human immunodeficiency virus type 1; TAR, *trans*-activation-responsive region; LTR, long terminal repeat; PCAF, p300/CBP-associating factor; RT, reverse transcriptase.

K28Q, pTat-K29R, pTat-K50R, and pTat-K50Q were generated by the site-directed mutagenesis method using the QuikChange kit (Stratagene). Mutated clones were fully sequenced after identification. All proviral constructions were derived from the pNL4-3 infectious molecular clone (43). pNL4-3 Tat(−) was generated by introducing two consecutive stop codons at amino acids 11 and 12. The plasmid was designated pNLT(−). To generate pNLT(−) that expresses wild-type or mutant Tat, wild-type or mutated *tat* coding sequence was introduced into the *nef* gene of pNLT(−) (44, 45). The resultant molecular genomes were designated pNLT, pNLT-K28Q, pNLT-K28R, pNLT-K29R, pNLT-K50Q, and pNLT-K50R.

Transfection and Infection—CEM cells were grown in RPMI 1640 medium (Invitrogen) supplemented with 10% FBS and antibiotics. 293, HeLa, and HeLa P4 cells, that contain the *lacZ* gene under control of the integrated HIV-1 LTR (46), were propagated in Dulbecco's modified Eagle's medium with 10% fetal bovine serum and transfected using calcium phosphate as described (40). Virus stocks were produced by transfection of 293 cells. Transfected cell supernatants were harvested at 48 h post-transfection and passed through 0.45-μm pore size filters. Viruses, normalized for reverse transcriptase (RT) activity, were used to infect CEM and HeLa P4 cells. Briefly, cells were incubated with virus for 2 h at 37 °C, washed, and resuspended in fresh medium. Virus production was monitored by RT assay of culture supernatants every 3 days.

RT and Reporter Assays—To measure RT activity, 10 μl of cell culture supernatants was mixed with 25 μl of RT buffer (60 mM Tris-HCl (pH 8), 75 mM KCl, 5 mM $MgCl_2$, 0.1% IGEPAL CA-630, 1.04 mM EDTA, 5 μg/ml poly(A), 0.16 μg/ml oligo(dT), 40 mM dithiothreitol, and 10 μCi/ml [α-^{32}P]dTTP (Amersham Biosciences)). The reactions were incubated for 2 h at 37 °C, and 10 μl was spotted onto a DEAE filter, washed three times in 2× SCC, dried, and quantified using an Instant Imager (Packard). To assay luciferase activity, transfected HeLa cells were lysed and assayed for luciferase activity 48 h post-transfection, according to the manufacturer's protocol (Promega). β-galactosidase activity was measured in extracts of HeLa P4 cells 48 h post-transfection or 24 h postinfection, according to the manufacturer's protocol (Roche Molecular Biochemicals).

In Vitro TAR/Tat Binding Assay—Wild-type and mutant Tat proteins were translated *in vitro* in a coupled transcription-translation rabbit reticulocyte lysate system (Promega) in the presence of [^{35}S]methionine according to the manufacturer's protocol. Synthetic biotinylated TAR RNA (2 μg) was immobilized on streptavidin-agarose beads and incubated with translated proteins for 2 h at 4 °C. The complex was then washed, resolved by SDS-PAGE, and analyzed by autoradiography.

Immunological Techniques and Western Blot Analysis—HeLa cells were transfected with the indicated plasmids. At 24 h post-transfection, cells were washed twice in phosphate-buffered saline and lysed in ice-cold lysis buffer (50 mM Tris-HCl (pH 8), 120 mM NaCl, 5 mM EDTA, 0.5% IGEPAL CA-630, 1 mM dithiothreitol, and protease inhibitor mixture). The cell lysates were clarified by centrifugation at 15,300 × *g* for 5 min, and supernatants were subjected to immunoprecipitation with the indicated antibody following a preclearing step. Immunoprecipitates were then washed three times with lysis buffer and resolved by SDS-PAGE. Proteins were transferred to polyvinylidene difluoride membrane using semidry blotting (Bio-Rad). Membranes were incubated with the primary antibody overnight at 4 °C, washed, and incubated for 1 h with the appropriate secondary antibody (Amersham Pharmacia Biotech). Proteins were visualized by chemiluminescence (Amersham Biosciences) according to the manufacturer's protocol. For immunofluorescence, HeLa cells were transfected with plasmids expressing FLAG-tagged Tat wild type or mutants. Cells were fixed 24 h after transfection with 4% paraformaldehyde for 10 min at room temperature. The cells were then washed and permeabilized in 1× phosphate-buffered saline containing 5% fetal calf serum and 0.1% Triton X-100 for 10 min at room temperature. Cells were stained with anti-FLAG antibody followed by incubation with Texas Red-conjugated anti-mouse antibody.

RESULTS

Mutation of Tat Acetyl-acceptor Lysines Affects HIV-1 Replication—HIV-1 Tat is essential for virus replication and is a potent *trans*-activator of viral gene expression (20). We have recently shown that Tat lysine 28, within the activation domain, and lysine 50, within the RNA binding domain, are targeted for acetylation by PCAF and p300, respectively. Mutation of acetyl-acceptor residues Lys^{28} and Lys^{50} to alanine reduces Tat-mediated *trans*-activation of the HIV-1 promoter in transient transfection assays (40). Previous analysis has shown a discordance between residues that are important for virus replication and those important for *trans*-activation of a transiently transfected reporter gene under the control of the HIV-1 LTR (47). Thus, we investigated the role of Tat acetyl-acceptor lysines in HIV-1 replication. Lysine 28 and lysine 50 were mutated to either arginine or glutamine. As a control, lysine 29 that is not acetylated was also mutated to arginine. Using a previously described strategy (45), Tat wild-type or mutants were introduced in the *nef* frame of pNL4-3 Tat(−) in which the *tat* gene had been inactivated by engineering two consecutive stop codons at amino acids 11 and 12 (Fig. 1*A*). The different constructs were used to transfect 293 cells, which express the adenoviral proteins E1A and E1B that strongly activate HIV-1 LTR and complement the defect in gene expression in viruses lacking Tat (45). Biochemical analysis of the resultant virions was performed. Fig. 1*B* shows that expression of viral proteins from recombinant genomes encoding wild-type or mutant Tat was identical. Furthermore, FLAG-tagged wild-type and mutant Tat proteins were readily detected in cells transfected with each of the respective viral genomes (Fig. 1*C*). The reverse transcriptase activity/p24 ratio was identical for all the molecular clones engineered (Fig. 1*D*). Thus, the engineered recombinant pNL4-3 molecular clones are competent for expression of the HIV-1 structural proteins with a normal RT/p24 ratio when transfected into 293 cells.

We then analyzed the replication of recombinant pNLT(−) encoding wild-type or mutant Tat in a T-cell line. Viruses, produced from 293 cells transfected with pNLT or pNLT mutants, were normalized for RT activity and used to infect CEM cells. Infected cells were monitored for virus replication by measuring supernatant RT activity every 2 or 3 days over a period of 21 days. Tat wild-type and Tat K29R viruses showed the same replication kinetics with an RT peak at day 13 postinfection (Fig. 1*E*). However, the mutations K28R, K28Q, and K50Q imposed significant replication defects. Tat K50R virus showed a 4-day delay to peak virus production when compared with the recombinant Tat wild-type virus. These results show that the acetyl-acceptor lysines within Tat play an important role in virus replication.

Effect of Tat Acetyl-acceptor Lysines on Tat-mediated Activation of Integrated and Nonintegrated LTR—To assess how mutation of Tat acetyl-acceptor lysines (Lys^{28} and Lys^{50}) affects virus replication, we analyzed their effect on Tat-mediated *trans*-activation of an integrated and nonintegrated HIV-1 LTR. HeLa P4 cells that contain the *lacZ* gene under the control of the integrated HIV-1 LTR (46) were infected with recombinant pNLT viruses encoding either Tat wild-type or mutants. β-Galactosidase activity was monitored 24 h after infection. Fig. 2*A* shows that transcriptional activity of Tat K50Q, Tat K28R, and Tat K28Q on the integrated LTR was severely reduced. Tat K50R transcriptional activity was slightly (1.6-fold) higher than the wild type. Similar results were obtained when the Tat-expressing constructs were transfected into HeLa P4 cells (data not shown). Taken together, these results show a correlation between the lack of transcriptional activity of Tat K28R, K28Q, and Tat K50Q on an integrated LTR and their inability to support virus replication. In contrast, the Tat K50R mutant, which showed delayed replication kinetics, was found to have slightly enhanced *trans*-activation.

We then analyzed the transcriptional activity of Tat wild-type and mutants in transient transfection assays. HeLa cells were transfected with an LTR luciferase reporter gene either alone or with Tat expression plasmids as indicated (Fig. 2*B*).

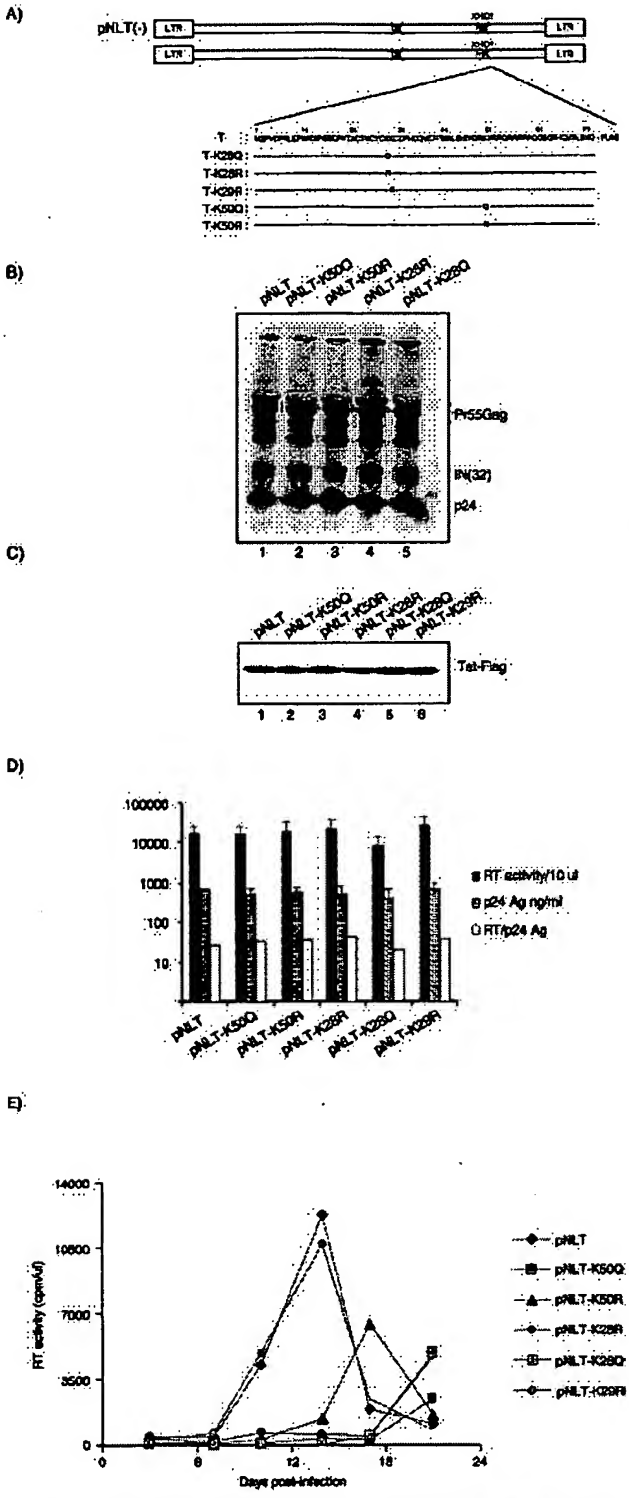

FIG. 1. **Analysis of pNL4-3 Tat(−) viruses that express wild-type or mutant Tat.** *A*, construction of pNL4-3 Tat(−) provirus engineered to express wild-type or mutant Tat. The pNL4-3 molecular genome was used as a backbone to generate pNL4-3 Tat(−), hereafter referred to as pNLT(−), by the introduction of two consecutive stop codons at amino acids 11 and 12. To generate pNLT(−) that expresses wild-type or mutant Tat, wild-type or mutated *tat* coding sequence was introduced into the *nef* gene of pNLT(−). The resultant molecular genomes were designated pNLT, pNLT-K28Q, pNLT-K28R, pNLT-K29R, pNLT-K50Q, and pNLT-K50R. *B*, biochemical analysis of pNLT(−) viruses expressing wild-type or mutant Tat. Western blot analysis was performed with equal quantities of virions produced from 293 cells transfected with pNLT(−) expressing wild-type or mutant Tat. HIV-1-specific proteins were detected using an HIV-1-neutralizing serum. *C*, Tat is efficiently expressed from recombinant pNLT(−) molecular genomes encoding wild-type or mutant Tat. Extracts of cells transfected with the indicated molecular genomes were analyzed by Western blotting using an anti-FLAG antibody. *D*, RT activity and p24 antigen of viruses produced by transfection of 293 cells with pNLT(−) expressing wild-type or mutant Tat. Data are averages from at least three independent assays ± S.E. *E*, Tat acetyl-acceptor lysines affect virus replication. Virus stocks obtained by transfection of 293 cells with the indicated molecular genomes were normalized for RT activity and used to infect CEM cells. RT activity in the cell supernatant was monitored over time.

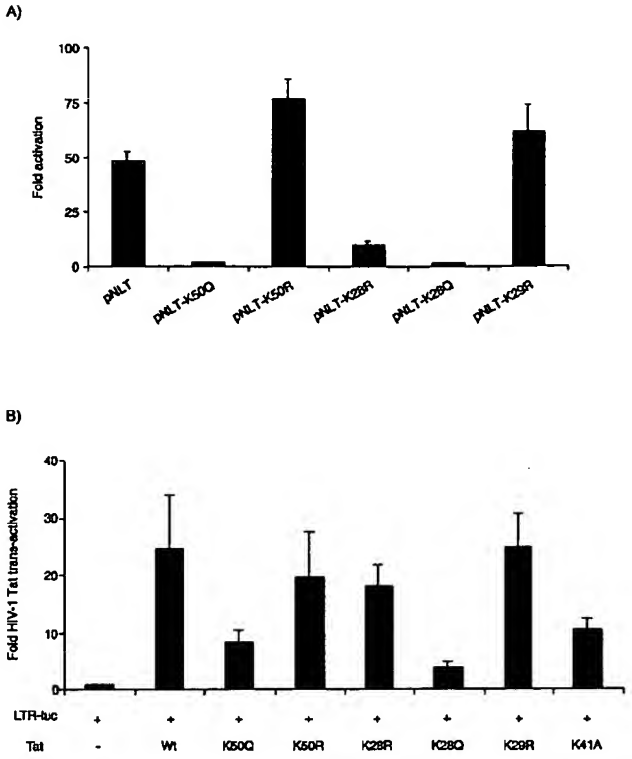

FIG. 2. **Tat acetyl-acceptor Lys^{28} and Lys^{50} are important for Tat-mediated *trans*-activation of the HIV-1 LTR.** *A*, virus stocks obtained by transfection of 293 cells with the indicated molecular genome were normalized for RT activity and used to infect HeLa P4 cells. Cell extracts were assayed for β-galactosidase activity 24 h postinfection. The results are presented as histograms indicating the induction of the integrated LTR by the indicated viruses with respect to the activity of cells infected with supernatant alone, which was assigned a value of 1. Data are averages from at least three independent assays ± S.E. *B*, HeLa cells were co-transfected with 1 μg of pLTR-luc wild type (*Wt*) and 0.1 μg of pRL-CMV either alone or together with 0.5 μg of plasmids expressing wild-type or mutated Tat as indicated. The relative luciferase activity was calculated following normalization for *Renilla* luciferase activity. Data are averages from at least three independent assays ± S.E.

The transcriptional activity of Tat K50Q and Tat K28Q was reduced (3- and 6.5-fold, respectively), while that of Tat K50R and Tat K29R was comparable with Tat wild type. Interestingly, the transcriptional activity of Tat K28R, which was reduced by 5-fold on an integrated LTR, was comparable with that of wild-type in transient transfection assays (1.35-fold reduction). As previously shown, the transcriptional activity of the Tat K41A was significantly reduced (22, 23). Taken together, the experiments shown in Fig. 2 suggest that the effect of Tat acetyl-acceptor lysines on Tat-mediated *trans*-activation is dependent on the promoter context. These experiments furthermore suggest that activation of an integrated *versus* nonin-

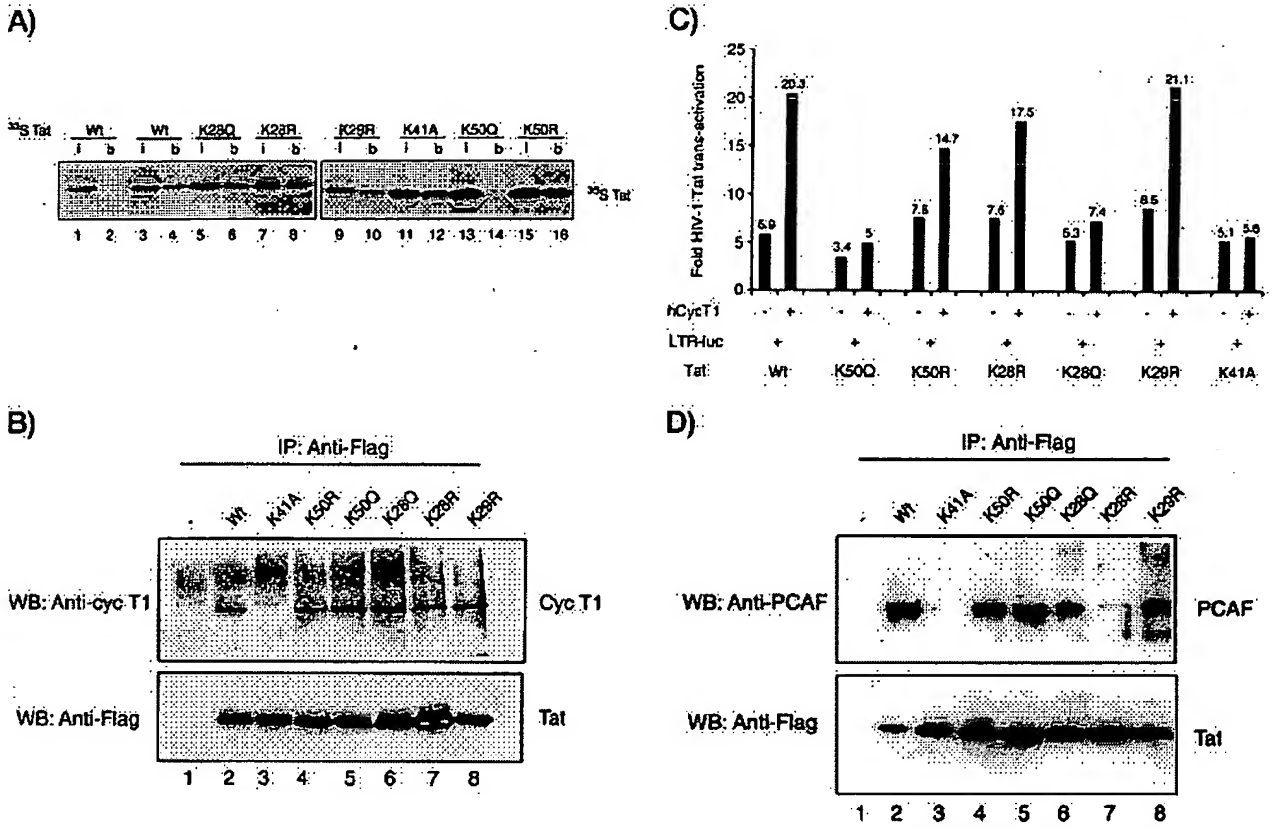

FIG. 3. **Acetyl-acceptor lysines are critical for Tat's interactions with TAR, cyclin T1, and PCAF.** *A*, *in vitro* translated ^{35}S-labeled wild-type (*Wt*) and mutant Tat were incubated with synthetic biotinylated TAR RNA (2 μg) or denatured TAR RNA (*lane 2*) that had been immobilized on streptavidin-agarose beads. Beads were washed, resolved by SDS-PAGE, and analyzed by autoradiography (bound (*b*)). A sample of the ^{35}S-labeled Tat proteins was analyzed directly (input (*i*)). *B*, HeLa cells were transfected with Tat-FLAG-expressing plasmids as indicated. Extracts were subjected to immunoprecipitation using anti-FLAG antibody, and immunoprecipitates were resolved by SDS-PAGE followed by immunoblotting using anti-cyclin T1 antibody (*upper panel*) or anti-FLAG antibody (*lower panel*). *C*, NIH 3T3 cells were co-transfected with 1 μg of pLTR-luc wild type, 0.1 μg of pRL-CMV, and 0.5 μg of plasmids expressing wild-type or mutant Tat, in the presence or absence of 0.5 μg of plasmid expressing human cyclin T1. The relative luciferase activity was calculated following normalization for *Renilla* luciferase activity expressed from the CMV promoter present in the pRL-CMV internal control plasmid. The fold HIV-1 Tat *trans*-activation was calculated relative to transfections performed in the absence of Tat expression plasmids. A representative experiment of three repeated transfections is shown. *D*, the experiment was performed as described for *B* except that immunoprecipitates resolved by SDS-PAGE were immunoblotted with anti-PCAF antibody (*upper panel*) or anti-FLAG antibody (*lower panel*). *WB*, Western blot; *IP*, immunoprecipitation.

tegrated HIV-1 LTR by Tat may involve different mechanisms.

Tat *trans*-activation of the HIV-1 LTR minimally requires TAR, positive transcription elongation factor b, and Tat-associated histone acetyltransferases. Thus, we analyzed the effect of Tat acetyl-acceptor mutants on the interaction between Tat and TAR, cyclin T1, and PCAF. Fig. 3*A* shows that Tat wild type, Tat K28Q, Tat K28R, Tat K29R, and Tat K50R interact with TAR RNA. However, a weak interaction was observed between Tat K50Q and TAR RNA. This finding suggests that the reduced *trans*-activation function of Tat K50Q is probably due to a loss of its interaction with TAR RNA.

Because the positive transcription elongation factor b complex is required for Tat *trans*-activation, we investigated the role of lysine 28 and 50 in the interaction of Tat with cyclin T1. HeLa cells were transfected with FLAG-tagged Tat wild type or mutants. 24 h after transfection, cell extracts were prepared and subjected to immunoprecipitation using anti-FLAG antibody. Immunoprecipitates were resolved on SDS-PAGE, and the presence of cyclin T1 was analyzed by immunoblotting using anti-cyclin T1 antibody. Fig. 3*B* shows that Tat wild-type and mutants were able to interact with cyclin T1. Tat K28R interaction with cyclin T1 was less efficient than Tat wild type (compare *lanes 2* and *7*). As previously reported, the Tat K41A mutant failed to interact with cyclin T1 (*lane 3*). We then analyzed the effect of human cyclin T1 on Tat-mediated *trans*-activation of the LTR in NIH 3T3 cells. As shown in Fig. 3*C*, human cyclin T1 enhanced Tat *trans*-activation by 3.4-fold. Although Tat K28Q and Tat K50Q were able to interact with cyclin T1, no synergistic activation of the LTR was observed. Human cyclin T1 enhanced Tat K28R and Tat K50R *trans*-activation by 2.3- and 1.9-fold, respectively. As expected, human cyclin T1 had no effect on Tat K41A transcriptional activity. These results suggest that binding of Tat to cyclin T1 is required but not sufficient for optimal *trans*-activation of the HIV-1 LTR.

Finally, because PCAF is known to assist Tat in activation of an integrated LTR (27), we analyzed the effect of Tat acetyl-acceptor lysines on the interaction between Tat and PCAF. Co-immunoprecipitation analysis showed that Tat wild type, Tat K50R, Tat K50Q, Tat K28Q, and Tat K29R were able to immunoprecipitate PCAF (Fig. 3*D*). However, Tat K41A and Tat K28R interacted weakly with PCAF. The expression level of Tat wild type and mutants is shown in the *lower panel* (Fig. 3*D*). This result may explain why Tat K28R is competent for activating the LTR in a transient transfection assay (Fig. 2*B*) but activates the integrated LTR poorly (Fig. 2*A*).

Because Tat K28Q and Tat K50Q are transcriptionally incompetent yet bind to cyclin T1 and PCAF, we asked whether

these mutants, by sequestering cyclin T1 and/or PCAF, are able to compete with Tat wild-type for LTR *trans*-activation. Thus, HeLa P4 cells were transfected with either Tat wild type alone or together with Tat mutants, and β-galactosidase activity was measured 24 h after transfection. As shown in Fig. 4, Tat K28Q, Tat K28R, and Tat K50Q reduced Tat wild-type transcriptional activity by 60, 40, and 70%, respectively. Tat K50R, Tat K29R, and Tat K41A showed no *trans*-dominant effect. Taken together, these experiments highlight the importance of cyclin T1 and PCAF in Tat-mediated *trans*-activation of the HIV-1 LTR.

Mutation of Tat Acetyl-acceptor Lysines Affects Its Subcellular Localization—Tat acetyl-acceptor lysines are localized to the activation domain (Lys^{28}) that mediates the interaction between Tat and its co-activators and the RNA binding domain (Lys^{50}) that also serves as a nuclear localization signal. Thus, we analyzed the effect of Lys^{50} and Lys^{28} mutations on the subcellular localization of Tat by immunofluorescence (Fig. 5). HeLa cells were transfected with either FLAG-tagged Tat wild type or mutants as indicated, and cells were stained with anti-FLAG antibody. Tat wild type showed a characteristic pattern consisting of diffuse nucleoplasmic fluorescence with intense nucleolar staining in 78% of transfected cells as observed previously (48, 49), whereas only 22% of transfected cells showed both nuclear and cytoplasmic staining. Tat K50Q was found to localize to both cytoplasm and nucleus in 90% of transfected cells. Tat K50R localized exclusively in the nucleus in 93% of transfected cells. Thus, the positive charge of Lys^{50} plays an important role in dictating Tat localization. Tat K29R showed a similar staining pattern to Tat wild-type. Mutation of Lys^{28} to glutamine increased the number of cells that showed both nuclear and cytoplasmic staining of Tat to 44%. Interestingly, Tat K28R, Tat K28Q, and Tat K41A showed a perinucleolar, instead of diffuse nucleolar, localization as seen for the Tat wild type, Tat K50R, and Tat K29R. These results suggest that Lys^{41} and acetyl-acceptors Lys^{28} and Lys^{50} of Tat influence its subcellular localization.

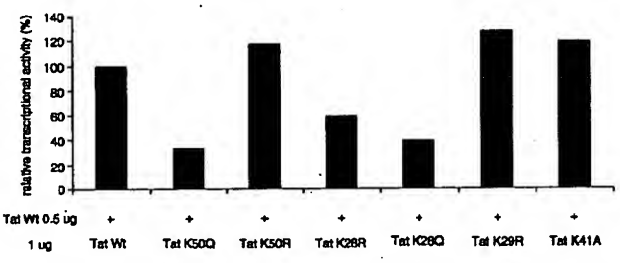

FIG. 4. **Tat acetyl-acceptor mutants show a *trans*-dominant effect on Tat wild-type transcriptional activity.** Extracts of HeLa P4 cells, transfected with wild-type (*Wt*) Tat alone or together with Tat mutants as indicated, were assayed for β-galactosidase activity 24 h post-transfection. A representative experiment of three repeated transfections is shown.

DISCUSSION

The HIV-1 *trans*-activator Tat is absolutely required for virus replication and plays a critical role in AIDS pathogenesis. In this report, we have analyzed the role of acetyl-acceptors Lys^{28} and Lys^{50} in virus replication and Tat transcriptional activity. Thus, lysines 28 and 50 were mutated to either arginine (to conserve the positive charge) or glutamine (to neutralize the positive charge). By introducing these mutations in the pNL4-3 Tat minus background, using a previously described strategy (45, 47), we show that acetyl-acceptor Lys^{28} and Lys^{50}

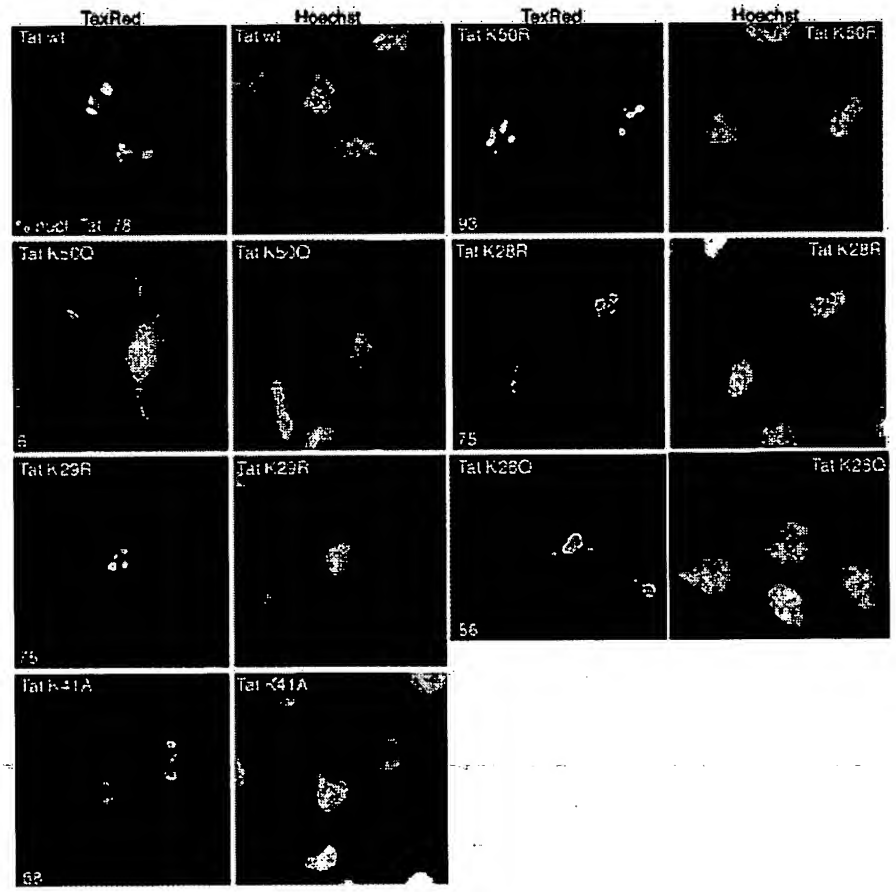

FIG. 5. **Tat acetyl-acceptor mutants show an altered subcellular localization.** HeLa cells were transfected with the indicated Tat-FLAG expression plasmids and stained with anti-FLAG antibody followed by Texas Red-conjugated anti-mouse antibody. *wt*, wild type.

play a critical role in virus replication. Mutation of Lys^{28} or Lys^{50} to glutamine results in replication-incompetent virus and transcriptionally inactive Tat on integrated and nonintegrated LTR. Interestingly, mutation of lysine 50 to arginine did not affect Tat-mediated *trans*-activation of either integrated or nonintegrated LTR but led to a 4-day delay in virus replication. Mutation of lysine 28 to arginine affected virus replication and activation of an integrated LTR without affecting LTR *trans*-activation in a transient transfection assay.

To investigate how mutation of Tat acetyl-acceptor lysines affects Tat transcriptional activity, we analyzed the effect of these mutations on Tat/TAR, Tat/cyclin T1, and Tat/PCAF interactions. Consistent with our previous report (40), mutation of lysine 50 to glutamine reduced the ability of Tat to bind TAR RNA without affecting its interaction with cyclin T1 or PCAF. Moreover, Tat K50Q has an altered cellular localization with nuclear and cytoplasmic staining in 90% of transfected cells. Thus, the lack of binding of Tat K50Q to TAR RNA and its abnormal cellular localization probably contribute to its inability to support virus replication and *trans*-activation of the LTR.

Tat K28R is able to interact with TAR and cyclin T1 but failed to interact with PCAF, one of the histone acetyltransferases that has been shown to assist Tat-mediated *trans*-activation of integrated LTR (27). Previously, it has been shown that the two-exon form of Tat activates an integrated LTR more efficiently than the Tat one-exon form (50). In the context of chromatin, Tat also has to overcome the chromatin repression exerted by the nucleosomal architecture of the integrated provirus (51, 52). In this respect, Tat has been shown to disrupt the repressive nucleosome 1 (nuc1) to activate the transcription from integrated LTR (52). Thus, the inability of Tat K28R to activate an integrated LTR and, consequently, the reduced replication of viruses carrying this mutation may be due to the loss of its interaction with PCAF. Taken together, these results suggest a fundamental difference in the mechanism by which Tat *trans*-activates an integrated *versus* nonintegrated LTR.

Mutation of Tat Lys^{28} to glutamine had no effect on Tat/TAR, Tat/cyclin T1, and Tat/PCAF interaction. Despite this, Tat K28Q was transcriptionally inactive on both integrated and nonintegrated LTR. Tat K28Q showed an altered subcellular localization with 44% of transfected cells stained in both cytoplasm and nucleus. The inability of Tat K28Q to activate the LTR and consequently to support virus replication may be explained, at least in part, by its altered subcellular localization. Additionally, K28Q mutation may affect the interaction between Tat and other cellular factors involved in *trans*-activation of the LTR and virus replication.

Mutation of Tat K50R did not affect its interaction with TAR, cyclin T1, or PCAF. Tat K50R activated both integrated and nonintegrated LTRs as efficiently as Tat wild type. However, K50R mutation delayed virus replication by 4 days. Transcriptionally competent Tat mutants unable to support optimal virus replication have been reported previously (47). How could transcriptionally active Tat fail to support optimal virus replication? Besides its primary function as a *trans*-activator of the viral promoter, Tat has been shown to have pleiotropic effects on cellular genes and metabolism (20). Tat is a secreted protein that is taken up by neighboring cells (2, 3). It has been shown that soluble Tat protein can activate noninfected cells, thus preparing a favorable cellular environment for virus replication (53). Soluble Tat is able to activate transcription factors, such as NF-κB, that in turn activate the HIV-1 promoter (54–56). Tat also plays an important role in reverse transcription of the viral RNA (1). Thus, the K50R mutation within Tat may affect its nontranscriptional activities and, consequently, virus replication. Consistent with this hypothesis, only 7% of transfected cells showed both cytoplasmic and nuclear localization of Tat K50R compared with 22% of Tat wild type-transfected cells, suggesting a defect in its secretion. Taken together, these results suggest that the transcriptional activity of Tat is necessary but not sufficient to support virus replication.

Accumulating evidence suggests that Tat may also be an important virulence factor *in vivo*. Vaccination of nonhuman primates with Tat, either alone or in combination with other viral products, reduces virus replication (6, 57–61). Whereas native Tat protein is cytotoxic, a modified Tat protein is considered an attractive target for HIV vaccine development. Thus, identification of Tat mutants able to compete with and inhibit wild-type Tat function will help to engineer the optimal Tat protein vaccine candidate.

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Transcriptional synergy between Tat and PCAF is dependent on the binding of acetylated Tat to the PCAF bromodomain

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The human immunodeficiency virus (HIV) Tat protein plays an essential role in promoting efficient transcriptional elongation of viral transcripts. We report that the transcriptional co-activator PCAF and Tat interact and synergize to activate the HIV promoter. The binding of Tat and PCAF *in vitro* and *in vivo* is dependent on the acetylated state of Lys50 of Tat and on the PCAF bromodomain. Structural analysis of the acetylated Tat peptide bound to the PCAF bromodomain defined amino acids Y47 and R53 in Tat and V763, Y802, and Y809 in PCAF as critical interaction points between the two proteins. Mutation of each of these residues in either Tat or PCAF inhibited in a cumulative manner the Tat–PCAF interaction *in vitro* and *in vivo*, and abrogated the synergistic activation of the HIV promoter by both proteins. These observations demonstrate that acetylation of Tat establishes a novel protein–protein interaction domain at the surface of Tat that is necessary for the transcriptional activation of the HIV promoter.

Keywords: acetylation/bromodomain/HIV/PCAF/Tat

Introduction

The Tat protein of human immunodeficiency virus (HIV) is a unique viral transactivator that binds to the Tat-responsive element (TAR), an RNA stem–loop structure that forms at the 5′ extremity of all viral transcripts (Cullen, 1998; Karn, 1999). In the absence of Tat, HIV transcription is highly inefficient because the assembled RNA polymerase II complex (RNAPII) cannot elongate efficiently on the viral DNA template (Garber and Jones, 1999). The binding of Tat to TAR stimulates the production of full-length HIV transcripts, and the integrity of the Tat/TAR axis critically determines the dynamics of viral replication in infected cells.

Recent experiments have defined the role of Tat in HIV transcription as an adaptor that co-ordinates the recruitment of critical co-factors near the transcription start site in the HIV promoter. Distinct functional domains of Tat are involved in the recruitment of different co-factors. The N-terminal cysteine-rich region interacts directly with the cyclin T1/cyclin-dependent kinase 9 (CDK-9) complex (Wei *et al.*, 1998). Recruitment of CDK-9 is thought to lead to hyperphosphorylation of the C-terminal domain of RNAPII and increased elongation efficiency. The C-terminal arginine-rich motif (ARM) in Tat is essential for RNA binding and nuclear localization. A single lysine residue in the ARM, K50, becomes acetylated by the transcriptional co-activator p300 *in vitro* and *in vivo* (Kiernan *et al.*, 1999; Ott *et al.*, 1999). Mutation of K50 to arginine, a mutation that conserves the local charge but prevents acetylation by p300, markedly decreases the synergistic activation of the HIV promoter by Tat and p300 (Ott *et al.*, 1999).

Reversible acetylation of lysine residues was first identified in histone proteins and plays an essential role in transcriptional regulation. Non-histone proteins including the transcriptional regulators $TF_{II}E$, $TF_{II}F$, p53, EKLF, GATA-1, HMGI(Y), HMG17, ACTR, MyoD and E2F1 are also reversibly acetylated (for a review see Kouzarides, 2000). In the case of chromatin, the level of acetylation of distinct lysine residues in each histone protein is under the control of competing histone acetylases (HATs) and histone deacetylases. Histone hypoacetylation is generally associated with transcriptional repression, while histone hyperacetylation has been correlated with transcriptional activation. Early models proposed that histone acetylation leads to a global neutralization of positive charges on histones and loosening of the histone–DNA interaction at transcriptionally active sites. However, recent data suggest that acetylated lysine residues on histone tails serve as a recognition code for the co-ordinated recruitment of specific factors (the 'histone code' hypothesis; Strahl and Allis, 2000). According to this model, acetylated lysine residues in the histone tails interact with a specialized protein module, the bromodomain (Dhalluin *et al.*, 1999; Jacobson *et al.*, 2000). Bromodomains are present in a variety of proteins, including nuclear HAT proteins, kinases and chromatin remodeling factors (Jeanmougin *et al.*, 1997).

To test the possibility that acetylated Tat also interacts with a bromodomain, we tested a variety of bromodomain-containing proteins for potential interaction with the acetylated ARM peptide of Tat. We observed that PCAF, a transcriptional co-activator containing a bromodomain, interacted specifically with acetylated Tat (Mujtaba *et al.*, 2002).

The goal of this study was to investigate the Tat–PCAF interaction and to examine its relevance to the transcrip-

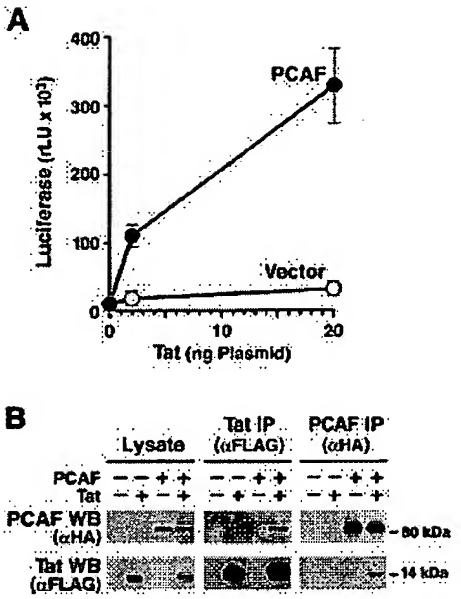

Fig. 1. Functional synergy and physical interaction between HIV Tat and PCAF. (A) Synergistic activation of the HIV promoter by Tat and PCAF. A PCAF expression vector (HA-PCAF) (Yang *et al.*, 1996b) or a control empty vector was co-transfected with an HIV Tat expression plasmid (Tat-FLAG) (Ott *et al.*, 1999) and the LTR-luciferase reporter construct into HeLa cells. Cells were harvested after 24 h, and the luciferase activity was measured. Luciferase values represent the mean ± SEM of three independent experiments. (B) Tat and PCAF co-immunoprecipitate from cellular lysates. HEK 293 cells were co-transfected with HA-PCAF and Tat-FLAG expression vectors (+) or the corresponding empty vector controls (–). In the left panel, cellular lysates analyzed by western blot (WB) show equal protein expression. Equal amounts of protein (500 μg/lane) were subjected to immunoprecipitation (IP) with the indicated antibodies and protein G–Sepharose. The immunoprecipitated material was analyzed by western blotting and antisera specific for the FLAG and HA epitopes (middle and right panels).

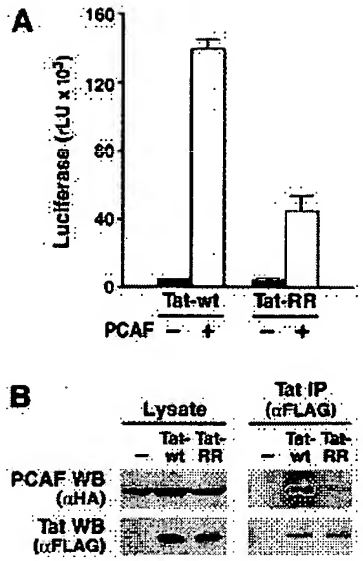

Fig. 2. Tat K50 is important for the synergy with PCAF and for Tat binding to PCAF. (A) Tat expression vector (Tat-wt or Tat-RR, in which K50 and K51 were replaced with arginine) was co-transfected with a PCAF expression vector and an LTR-luciferase reporter in HeLa cells. Luciferase values are the mean ± SEM of three independent transfection experiments. (B) Expression vectors for Tat-wt and Tat-RR (both FLAG tagged) were co-transfected with PCAF-HA. In the left panel, cellular lysates analyzed by western blot (WB) show equal protein expression. Equal amounts of protein (500 μg/lane) were subjected to immunoprecipitation (IP) with the anti-FLAG antiserum and protein G–Sepharose. The immunoprecipitated material was analyzed by western blotting and antisera specific for the FLAG and HA epitopes (right panel).

tional activation process mediated by Tat. We demonstrate that Tat and PCAF interact specifically via the ARM domain of Tat and the bromodomain of PCAF *in vitro* and *in vivo*. This interaction is critically dependent on the acetylation of K50 in Tat. Tat and PCAF functionally synergize to activate the HIV promoter and amino acids that are critical for the interaction *in vitro* and *in vivo* are also important for the transcriptional activity of the Tat protein.

Results

Tat and the transcriptional co-activator PCAF interact functionally to activate the HIV promoter

The identification of the bromodomain of PCAF as a protein module that specifically recognizes the acetylated ARM peptide (Mujtaba *et al.*, 2002) suggested that PCAF might play a role in the transcriptional activation of the HIV promoter. To test this possibility, we co-transfected expression vectors for PCAF and Tat and measured their combined effect on the activity of the HIV promoter. The HIV promoter was activated synergistically in response to Tat and PCAF (Figure 1A). This synergy was particularly striking at low Tat concentrations and decreased at higher Tat plasmid concentrations (not shown). This synergy was specific for the HIV promoter and was not observed when another retroviral promoter, from Rous sarcoma virus, was co-transfected (data not shown).

To determine whether Tat and PCAF can interact within cells, we co-transfected an expression vector for an epitope (FLAG)-tagged Tat protein with a hemagglutinin (HA)-tagged PCAF expression vector. Western blot analysis of lysates with antisera for FLAG and HA demonstrated comparable expression levels of both proteins after transfection (Figure 1B). Cellular extracts of transfected cells were subjected to immunoprecipitation with antisera specific for the FLAG or HA epitope. The immunoprecipitated material was analyzed by western blotting analysis for both Tat and PCAF. PCAF co-immunoprecipitated with Tat in both directions. These experiments suggested that both proteins stably interact within cells and that this interaction might be important for their synergistic activation of the HIV promoter.

Tat K50 and K51 are necessary for functional synergy and interaction between Tat and PCAF

To test our original hypothesis that Tat binding to PCAF is mediated, at least in part, by the specific recognition of acetylated Tat by the bromodomain of PCAF, we replaced Tat residues K50 and K51 with arginines. This amino acid substitution blocks acetylation and conserves the local charge environment in the Tat protein. Comparison of the transcriptional activities of wild-type Tat (Tat-wt) and the Tat mutant (Tat-RR) showed that inhibition of Tat acetylation decreased the synergy between Tat and

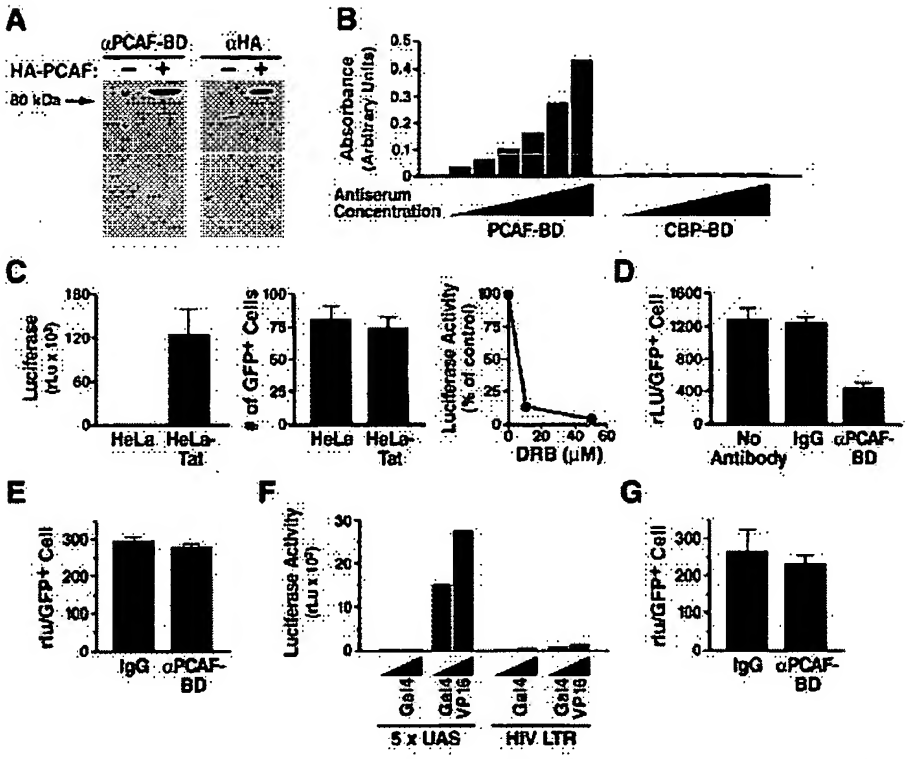

Fig. 3. Inhibition of Tat transactivation by an antiserum specific for the PCAF bromodomain. (**A**) Western blot analysis of cellular lysates (50 μg/lane) of HEK 293 cells transfected with HA-PCAF with an antiserum specific for the PCAF bromodomain (αPCAF-BD) (1:100). The same membrane was probed with an anti-HA antiserum as control to visualize PCAF-HA (αHA). (**B**) Purified anti-PCAF bromodomain IgGs recognize recombinant PCAF bromodomain (PCAF-BD) and not the closely related CBP bromodomain (CBP-BD) proteins. Bromodomain proteins (500 ng of protein) were coated onto polystyrene plates and developed by ELISA with different concentrations of the anti-PCAF bromodomain IgGs (10–0.3 mg/ml). (**C**) HeLa cells stably expressing Tat protein (HeLa-Tat) and standard HeLa cells were microinjected with the HIV LTR-luciferase reporter. Another construct containing GFP under the control of the CMV promoter was co-injected as a control. Four hours post-injection, GFP-positive cells were counted, and cells were harvested for luciferase activity measurements. Average luciferase values (relative light units rLU ± SEM; left panel) and average number of GFP-positive cells (± SEM; middle panel) from five independent experiments are shown. In the right panel, HeLa-Tat cells microinjected with the HIV LTR-luciferase construct were treated with 0, 10 or 50 μM 5,6-dichlorobenzimidazole riboside (DRB), an inhibitor of the Tat-associated CDK-9, and luciferase activity was measured. (**D**) Anti-PCAF BD or pre-immune IgGs (5 μg/ml) were microinjected into HeLa-Tat cells with the HIV LTR-luciferase reporter construct, and luciferase values were measured as described above. Luciferase values are the mean ± SEM of three independent experiments. (**E**) Anti-PCAF BD or pre-immune IgGs (5 μg/ml) were pre-incubated with recombinant PCAF BD *in vitro*. The resulting mixture was microinjected into HeLa-Tat cells with the HIV LTR-luciferase reporter construct, and luciferase values were measured as described above. Luciferase values are the mean ± SEM of three independent experiments. (**F**) Constructs corresponding to 5× Gal4 binding site upstream of the TK promoter (Puigserver *et al.*, 1999) or the HIV LTR both driving the luciferase reporter gene were microinjected in HeLa cells with either a plasmid encoding Gal4 or a Gal4–VP16 fusion protein. A representative experiment is shown. (**G**) A plasmid corresponding to 5× Gal4 binding site upstream of the TK promoter (Puigserver *et al.*, 1999) was microinjected in HeLa cells with a vector encoding the Gal4–VP16 fusion protein in the presence of anti-PCAF BD or pre-immune IgGs (5 μg/ml). Luciferase values were measured as described above. Luciferase values are the mean ± SEM of three independent experiments.

PCAF by 70% (Figure 2A). In agreement with the functional data, we observed that mutation of K50 and K51 partially suppressed the interaction between Tat and PCAF *in vivo* (Figure 2B).

Inhibition of Tat transactivation of the HIV promoter by an antiserum specific for the bromodomain of PCAF

To further assess the role of the PCAF bromodomain in Tat activity, we raised a polyclonal antiserum using a recombinant PCAF bromodomain expressed in *Escherichia coli*. This antiserum specifically recognized PCAF by western blot in cellular extracts, and did not cross-react with cellular proteins (Figure 3A). It also exhibited high specificity, since the closely related bromodomain from the transcriptional co-activator CBP was not recognized, even when the antiserum was used at high concentration in an ELISA (Figure 3B).

Next, we established a nuclear microinjection assay to measure Tat transactivation quantitatively. HeLa cells stably expressing a full-length Tat protein (HeLa-Tat) or control HeLa cells were microinjected with a construct in which the luciferase reporter is under the control of the HIV promoter. A construct expressing enhanced green fluorescent protein (GFP) under the control of the cytomegalovirus promoter (CMV-GFP) was co-injected to assess the number and viability of injected cells before harvest. In a representative experiment, luciferase values were 500-fold higher in HeLa-Tat cells than in control HeLa cells (Figure 3C). On average, 100 cells were injected per condition, and the survival rate was ~75% in both cell lines (Figure 3C, middle panel). In agreement

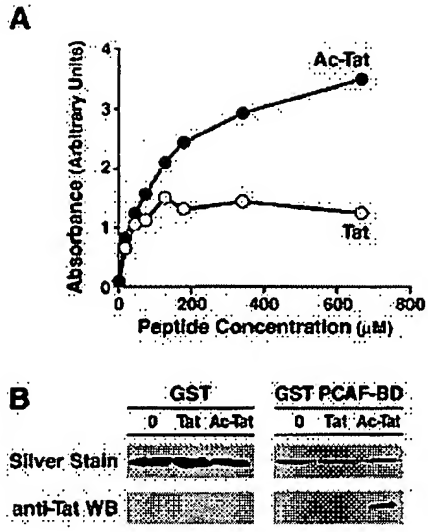

Fig. 4. PCAF bromodomain directly interacts with acetylated Tat *in vitro*. (A) An ELISA was used to demonstrate direct binding of the ARM peptide of Tat with recombinant bromodomain protein. Plates were coated with the bromodomain and incubated with biotinylated ARM peptide corresponding to Tat amino acids 43–58 containing either acetylated or unacetylated Tat. The binding of the peptide to the plate was measured with streptavidin–HRP and a colorimetric assay. A representative experiment is shown (duplicates). (B) Recombinant GST–PCAF bromodomain or GST protein was incubated with fully synthetic Tat proteins (non-acetylated or acetylated on K50). GST–PCAF and GST proteins were bound to glutathione beads, washed and eluted in Laemmli buffer. Eluted proteins were analyzed by silver staining to visualize the eluted GST and GST PCAF BD proteins, and by western blotting with an antiserum specific for Tat.

with previous observations, Tat transactivation in this system was inhibited in a dose-dependent manner by 5,6-dichlorobenzimidazole riboside (DRB), a CDK-9 inhibitor that blocks Tat function (Mancebo *et al.*, 1997; Figure 3C, right panel). Microinjection of an antiserum specific for the bromodomain of PCAF inhibited Tat-mediated transactivation of the HIV promoter in this system, whereas microinjection of the pre-immune serum or no antibody had no effect (Figure 3D). Additional control experiments were performed to ensure the specificity of this inhibition. First, we observed that pre-incubation of PCAF BD antiserum with an excess of recombinant PCAF BD protein prior to microinjection blocked the suppressive effect of this antiserum on HIV transcription (Figure 3E). Secondly, we tested the effect of the PCAF BD antiserum on another Tat-independent promoter. We used a plasmid containing five Gal4 binding sites upstream of the thymidine kinase (TK) promoter (Puigserver *et al.*, 1999). This promoter can be induced transcriptionally by a fusion protein between the DNA binding domain of Gal4 and the transactivating domain of VP16, but not by the DNA binding domain of Gal4 alone (Figure 3F). Microinjection of the anti-PCAF BD antiserum had no effect on the transactivation mediated by the Gal4–VP16 fusion protein (Figure 3G). These experiments demonstrate that the bromodomain of PCAF and the ARM region of Tat are important for the transactivation of the HIV promoter and are consistent with a direct interaction between Tat and PCAF mediated by the bromodomain of PCAF and acetylated K50 of Tat.

Direct interaction between acetylated Tat and the bromodomain of PCAF in vitro

As discussed above, preliminary experiments using a GST–PCAF bromodomain fusion protein indicated that a Tat peptide corresponding to the ARM region of Tat (GGLGISYGRKKRRQRRRP) could bind directly to the recombinant PCAF bromodomain.

We demonstrated independently the ability of Tat to interact directly with the PCAF bromodomain with two distinct biochemical approaches. First, we used the recombinant PCAF bromodomain to coat microwell plates and tested its ability to bind a biotinylated peptide corresponding to amino acids 41–58 of Tat. Reactions were developed colorimetrically after incubation with streptavidin–horseradish peroxidase (HRP). We detected a dose-dependent binding of K50-acetylated Tat peptide. In contrast, the same Tat peptide containing unacetylated K50 showed lower, dose-independent binding, consistent with a non-specific interaction (Figure 4A). To determine whether full-length Tat also interacts with the PCAF bromodomain, we used a GST–PCAF bromodomain fusion protein and two full-length synthetic Tat proteins, one unacetylated and the other acetylated at K50. Both proteins activated the HIV promoter transcriptional activity after microinjection into HeLa cells (M.Ott and E.Verdin, unpublished observations), and the chemical synthesis ensured the biochemical homogeneity of the protein preparations. Both proteins were incubated either with purified GST or GST–PCAF bromodomain proteins *in vitro*. K50-acetylated Tat bound to the GST–PCAF bromodomain but not to GST alone. This interaction was strictly dependent on the presence of an acetyl group on K50, since the unacetylated Tat protein did not bind to the PCAF bromodomain (Figure 4B).

Mutations in the PCAF bromodomain and the ARM domain of Tat cumulatively inhibit their interaction in vivo

The PCAF bromodomain structure consists of a left-handed, four-helix bundle (helices aZ, aA, aB and aC; Dhalluin *et al.*, 1999). Analysis of the Tat/PCAF bromodomain structure revealed that while the overall three-dimensional structure of the bromodomain was preserved, the ZA and BC loops, which compose the acetyllysine binding site, underwent significant conformational changes when bound to Tat. The Tat peptide adopted an extended conformation and lay across a pocket formed between the ZA and BC loops. The side-chain of the acetyllysine residue was located in the protein hydrophobic cavity and interacted extensively with several PCAF bromodomain residues, including V752, Y760, I764, Y802 and Y809. Peptide residues flanking Tat K50 also contacted the protein. Tat residues G48, R49 and R53 showed intermolecular nuclear Overhauser effects (NOEs) on the protein, while Y47 and Q54 formed extensive pairwise interactions with V763 and E756 of PCAF, respectively (for full description and discussion of these observations, see Mujtaba *et al.*, 2002). *In vitro* mutagenesis analysis of PCAF based on this structural information confirmed that residues Y809, Y802, V752 and F748 of PCAF were essential for acetyllysine binding, whereas V763 and E756 were important for recognition of Tat residues Y47, R53 and Q54. Together, these specific

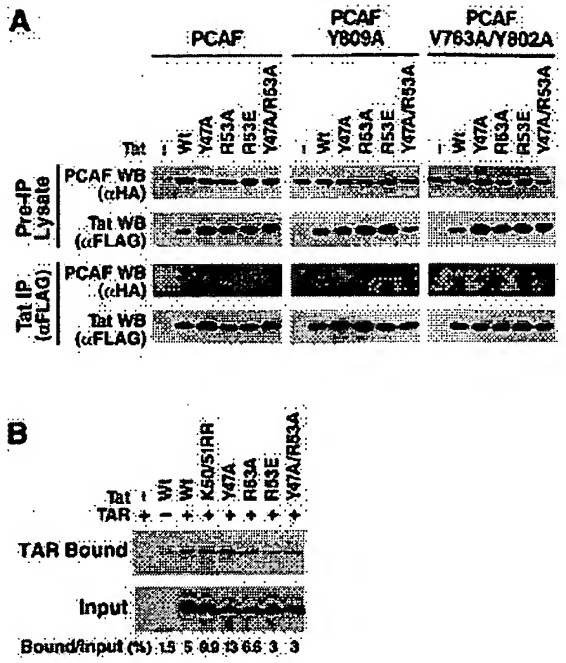

Fig. 5. Differential binding of Tat-wt and ARM mutants to the PCAF BD and to TAR RNA. (A) HA-PCAF and Tat-FLAG expression vectors (wild-type or mutant proteins) were transfected in HEK 293 cells. In the two upper panels, cellular lysates were directly assayed by western blotting (WB) with the indicated monoclonal antibodies (αHA for PCAF and αFLAG for Tat) to assess the level of protein expression after transfection (Pre-IP Lysate). In the lower two panels, cellular lysates were subjected to Tat immunoprecipitation with anti-FLAG antiserum, and the immunoprecipitated material was analyzed by western blot (WB) with the indicated monoclonal antibodies. (B) Binding of Tat-wt and ARM mutants to TAR. Tat and mutant Tat proteins were translated and biolabeled *in vitro*, incubated with a synthetic RNA corresponding to nucleotides 1–57 of TAR-biotin. After incubation, TAR and bound proteins was bound to streptavidin–agarose beads, centrifuged and washed. The bound proteins were eluted and analyzed by SDS–PAGE and autoradiography.

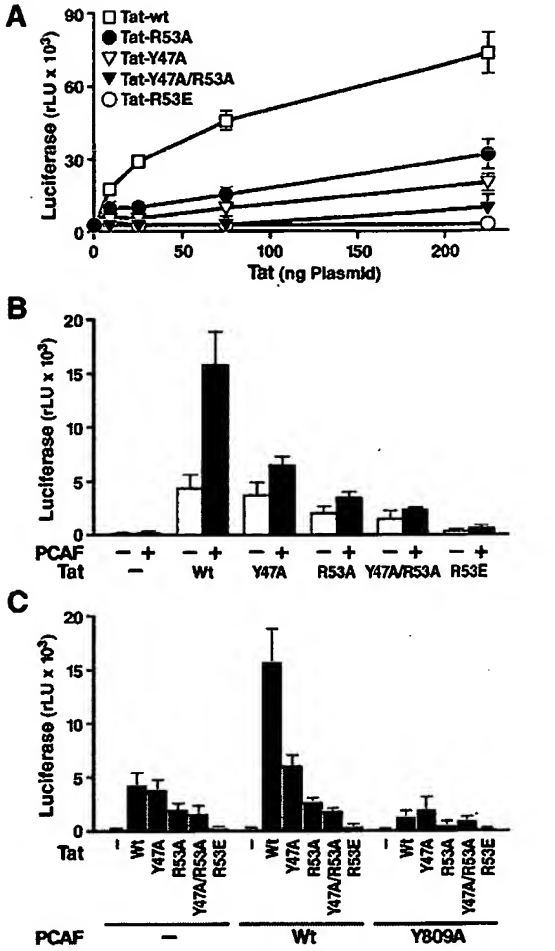

Fig. 6. Functional synergy between Tat and PCAF is disrupted by mutations that inhibit interactions between the PCAF bromodomain and acetylated Tat. (A) Tat-FLAG-expression vectors and mutants were fused to the NLS of the SV40 large T antigen and transfected into Jurkat cells with the LTR-luciferase reporter construct. Similar results were obtained with expression vectors for Tat proteins not fused to the SV40 large T antigen (data not shown). (B) Expression vectors for Tat and mutants and PCAF were co-transfected into HeLa cells with the LTR-luciferase reporter construct. Cells were harvested after 24 h, and luciferase activity was measured. Luciferase values are the mean ± SEM of three independent transfections. (C) Expression vectors for Tat and PCAF were co-transfected into HeLa cells with the LTR-luciferase reporter construct. Cells were harvested after 24 h, and luciferase activity was measured. Luciferase values are the mean ± SEM of three independent transfections.

interactions result in a highly selective association between Tat and the bromodomain of PCAF.

To examine the *in vivo* relevance of these amino acids to the binding of Tat to PCAF *in vivo*, we introduced point mutations in PCAF at positions 809 (Y809A), and 763 and 802 (V763+Y802A) with the aim of destabilizing the Tat–PCAF interaction. We also introduced corresponding mutations in the Tat protein at amino acids that participate in the interaction with the bromodomain of PCAF (Y47A, R53A, R53E and Y47A+R53A). These constructs were co-transfected into HeLa cells, and the Tat–PCAF interaction was tested after immunoprecipitation of Tat. All recombinant proteins, Tat and PCAF, wild-type and mutants, were expressed at the same level after transfection (Figure 5A). Tat-wt immunoprecipitated PCAF and PCAF-bromodomain mutants (V763+Y802A and Y809) to nearly the same extent (Figure 5). Introduction of mutations in Tat in amino acids involved in bromodomain recognition (Y47A, R53A, R53E and Y47A+R53A) significantly reduced the ability of Tat to immunoprecipitate wild-type PCAF (Figure 5). Combining Tat mutations with PCAF mutations completely abolished the Tat–PCAF interaction (Figure 5). This experiment confirmed the crucial role of the PCAF bromodomain and the Tat ARM domain in the PCAF/Tat interaction. Amino acids defined as critical by structural analysis and by *in vitro* binding assays are also critical for Tat–PCAF interaction *in vivo*.

Since the region targeted by our mutations is also involved in RNA binding of Tat to TAR, we assessed the effect of each mutation on TAR binding. *In vitro* translated Tat-wt was incubated with a synthetic TAR RNA incorporating a biotin label at its 5′ extremity. The TAR RNA was bound to agarose–streptavidin beads, centrifuged and the bound protein eluted by boiling in Laemmli buffer. The eluted material was analyzed by SDS–PAGE analysis followed by autoradiography. This analysis showed that

three mutants, K50/51RR, Y47A and R53A, showed relative increased binding to TAR. In contrast, the two remaining mutants, R53E and Y47A/R53A, showed moderately decreased binding (40%) in comparison with Tat-wt (Figure 5B).

Mutation that suppress the Tat–PCAF interaction in vitro and in vivo also inhibit Tat-mediated transactivation

To further define the role of the Tat–PCAF interaction in relation to the Tat-mediated transactivation process, we examined the effect of the same Tat and PCAF mutations on Tat transactivation. First, we examined the effect of these mutations without adding exogenous PCAF. Since the ARM region studied here is also the nuclear/nucleolar localization signal, mutations in this domain might interfere with the subcellular localization of Tat. To exclude this possibility, all transfections were performed both with Tat-FLAG constructs and with fusion constructs in which the nuclear localization signal of SV40 large T antigen was added to the N-terminus of Tat-FLAG (NLS-Tat). NLS-Tat protein was fully competent to activate the HIV promoter (Figure 6A), and all mutants in the ARM domain of Tat expressed as NLS fusion proteins were transported to the nucleus with the same efficiency, as determined by immunofluorescence microscopy (data not shown). Both individual and combined mutations in the ARM domain of Tat inhibited the transcriptional activity of NLS-Tat on the HIV LTR with the following efficiency: R53E > Y47A+R53A > Y47A > R53A (Figure 6A). Since a partial defect in RNA binding was noted for both the R53E and Y47A/R53A mutants, the defect in transactivation is difficult to interpret. However, the R53A and Y47A single mutants bound TAR with normal or increased affinity; therefore, the defect in transactivation is likely to occur as a result of defective PCAF binding. Similar results were observed when the same mutations were examined in the context of Tat protein not fused to the SV40 large T antigen NLS (data not shown).

Next, we examined the ability of each Tat mutant to synergize with PCAF. As shown above, co-transfection of Tat with wild-type PCAF led to synergistic activation of the HIV promoter (Figure 6B). Mutations in Tat decreased the synergy with wild-type PCAF in the same manner as in the absence of exogenous PCAF: R53E > Y47A+R53A > Y47A > R53A (Figure 6B). Finally, the effect of wild-type PCAF and mutant PCAF Y809A were compared both in the presence of Tat-wt and mutant Tat proteins (Figure 6C). This experiment showed that the PCAF Y809 mutation aggravated the defect caused by the Tat mutations alone. In fact, transfection of the PCAF mutant suppressed Tat-mediated transactivation in comparison to the control transactivation (–PCAF; Figure 6C). This observation suggest that PCAF Y809A functions as a dominant-negative mutant in Tat transactivation and suppresses partially the activity of endogenous wild-type PCAF.

Discussion

The discovery that the HIV Tat protein is modified by reversible acetylation raised new questions regarding the mechanism of Tat transactivation. Here, we present evidence that acetylated Tat specifically interacts with the transcriptional co-activator PCAF. This interaction is direct and mediated by the bromodomain of PCAF, a conserved protein module previously described to contain an acetyllysine binding pocket for histone peptides (Dhalluin *et al.*, 1999). Point mutations of amino acid residues Y47 and R53 in Tat or residues V763 and Y802 in PCAF severely impaired both the binding of Tat to PCAF *in vivo* and the transcriptional synergy observed between the two proteins on the HIV promoter. These findings support the model that the recruitment of PCAF by acetylated Tat plays an important role in the regulation of HIV transcription.

PCAF was originally identified as a factor that competes with E1A for binding to p300 (Yang *et al.*, 1996b). PCAF exhibits HAT activity (Ogryzko *et al.*, 1996) and resides in cells in multiprotein complexes containing other transcriptional regulatory proteins, including counterparts of the yeast ADA2, ADA3 and SPT3 proteins (Ogryzko *et al.*, 1998), histone-like TAFs (Ogryzko *et al.*, 1998) and the transcriptional co-activators p300 and ACTR/Src1 (Yang *et al.*, 1996b; Chen *et al.*, 1997). PCAF is also associated with the elongation-competent form of RNA polymerase II (Cho *et al.*, 1998) and plays an important role in several biological functions, including differentiation, cell cycle progression and gene-specific transcriptional regulation.

Although the acetylated ARM domain and the PCAF bromodomain were sufficient to mediate a Tat–PCAF interaction, other domains in each protein might further contribute to binding of the two proteins *in vivo*. Tat reportedly binds to p300 (Benkirane *et al.*, 1998; Hottiger and Nabel, 1998; Marzio *et al.*, 1998), which interacts with PCAF (Yang *et al.*, 1996b). However, we have been unable to detect p300 in PCAF and Tat co-immunoprecipitation experiments and conclude that p300 does not participate in the Tat–PCAF interaction. Benkirane *et al.* (1998) reported that PCAF interacts with Tat in GST pull-down experiments and synergizes with Tat to activate the HIV promoter. The HAT domain of PCAF was essential for PCAF-mediated activation of the HIV promoter, and this activation was only observed on chromatinized templates. Our observations confirm the functional synergy between Tat and PCAF and further demonstrate that the Tat–PCAF interaction *in vitro* and *in vivo* and their transcriptional synergy are mediated by the specific recognition of acetylated Tat by the PCAF bromodomain. In particular, the inhibition of Tat activity by microinjection of a specific anti-PCAF bromodomain antiserum supports the *in vivo* relevance of the observed Tat–PCAF bromodomain interaction.

The role of the PCAF HAT domain in the acetylation of histones or Tat remains to be investigated further. PCAF has been reported to acetylate K28 of Tat (Kiernan *et al.*, 1999). However, a Tat peptide containing acetylated K28 does not specifically interact with the PCAF bromodomain (Mujtaba *et al.*, 2002). We have also observed that recombinant PCAF acetylates Tat on K50 in the ARM domain with weak efficiency (W.Dormeyer, A.Dorr, M.Schnolzer and M.Ott, manuscript in preparation). However, given the marked discrepancy in the efficiency of Tat acetylation by p300 and PCAF, we do favor the hypothesis that Tat acetylation is mediated by p300.

The specific interaction of acetylated Tat with the bromodomain of PCAF could modulate the enzymatic activity of PCAF on histones or other substrates. Recently, another viral transactivator, the protein E1A of adenovirus, was shown to bind to several HAT proteins and to modulate their enzymatic and biological activities (Chakravarti *et al.*, 1999; Hamamori *et al.*, 1999). Such an effect of Tat on PCAF and other bromodomain-containing proteins could potentially explain the pleiotropic effects exerted by Tat on the expression of many cellular genes (reviewed in Rosenblatt *et al.*, 1995).

While the data presented here establish a potential mechanism for the Tat–PCAF synergy, it will be critical to determine at what step in the transactivating process this interaction takes place. Our working model is that Tat becomes acetylated after binding to TAR and coming into close contact with p300 bound to the HIV promoter (Marzio *et al.*, 1998; Ott *et al.*, 1999). According to this model, the interaction between Tat and PCAF is restricted to Tat associated with the HIV promoter. It is not entirely clear whether PCAF can interact with Tat bound to TAR or whether the Tat–PCAF interaction causes Tat to dissociate from TAR. *In vitro* titration experiments indicate that PCAF competes efficiently against TAR RNA for binding to the K50-acetylated Tat peptide (Mujtaba *et al.*, 2002). *In vitro* RNA gel shift experiments have also failed to show the binding of PCAF to TAR in the presence or the absence of Tat, whereas Tat bound to TAR efficiently (data not shown). These results imply that the PCAF bromodomain interaction with acetylated K50 on Tat may lead to the release of acetylated Tat from TAR RNA. Such dissociation could help in transferring Tat from TAR onto the elongating RNAPII, as reported previously (Wu-Baer *et al.*, 1995; Yang *et al.*, 1996a; Cujec *et al.*, 1997). PCAF specifically associates with the elongating form of RNAPII and is thought to play a role in the hyperacetylation of histones in transcribed domains of chromatin (Cho *et al.*, 1998). In contrast, p300 is associated with the hypophosphorylated, initiation-competent form of RNAPII (Cho *et al.*, 1998). It is conceivable that acetylated Tat helps in the loading of PCAF to the elongating polymerase (Wu-Baer *et al.*, 1995; Yang *et al.*, 1996a; Cujec *et al.*, 1997). According to this scenario, acetylated Tat could serve as a specific adaptor between PCAF and the elongation-competent RNAPII, thereby facilitating transcriptional elongation. Future experiments will test this hypothesis and should further increase our understanding of HIV transcriptional regulation by the transactivator protein Tat.

Materials and methods

Cells and plasmids

HeLa and HEK 293 cells were from the American Type Culture Collection, Jurkat 1G5 cells were obtained from Aguilar-Cordova and colleagues through the AIDS Research and Reference Reagent Program (Division of AIDS, NIAID, NIH). HeLa-Tat cells were a gift from P.Krammer, Heidelberg, Germany (Westendorp *et al.*, 1995). The N-terminally HA-tagged PCAF construct was amplified from the PCAF open reading frame (ORF) (Yang *et al.*, 1996b) with a 5′ primer containing the coding sequence for influenza HA and cloned into pCI (Promega). The full-length (101 amino acid) C-terminally FLAG-tagged Tat-wt and Tat-RR have been described previously (Ott *et al.*, 1999). HA-PCAF and Tat-FLAG constructs were used as templates for QuickChange site-directed mutagenesis with primers carrying the indicated mutations (Stratagene). After mutagenesis, the Tat ORF was fully sequenced. For PCAF, a *PvuII–KpnI* fragment containing the HAT region and the bromodomain was subcloned, sequenced and cloned back into the wild-type construct. The LTR-luciferase construct has been described previously (Emiliani *et al.*, 1996). The CMV-GFP construct was obtained from Clontech.

Transfections and luciferase assays

Initial co-transfections with Tat-, Tat-RR- and PCAF-expressing plasmids were performed in HeLa cells as described previously (Ott *et al.*, 1999). The co-transfections with Tat mutants and PCAF mutants were performed in HeLa cells with the calcium phosphate precipitation method. Cells were harvested 24 h after transfection of 1.5 μg of plasmid DNA per well (6-well plates) and analyzed for luciferase activity. Transient transfections into Jurkat 1G5 cells were performed with DEAE–dextran (225 ng of DNA/3 × 10^5 cells). Cells were harvested after 24 h, and luciferase activity was measured (Ott *et al.*, 1999).

Co-immunoprecipitations

HEK 293 cells were transfected in duplicate with expression vectors for Tat-FLAG, HA-PCAF, or empty vector constructs (total 1 μg of DNA) using 8 μl LipofectAMINE reagent (Invitrogen Life Sciences) for 6 h. After 24 h, cells were lysed in 250 mM NaCl, 0.1% NP-40, 20 mM NaH_2PO_4 pH 7, 5 mM EDTA, 30 mM sodium pyrophosphate, 10 mM NaF and protease inhibitor cocktail tablets (Roche). Duplicates were pooled and equal amounts of total protein were immunoprecipitated with anti-FLAG (M2; Sigma) or anti-HA (Roche) monoclonal antibodies (10 μg/ml each) together with protein G–Sepharose (Amersham Pharmacia) for 6 h at 4°C. Pellets were washed five times in lysis buffer, resuspended in Laemmli buffer, and analyzed by western blot with anti-FLAG (M2; Sigma) or anti-HA (Roche) monoclonal antibodies (10 μg/ml each).

Tat synthesis and purification

Solid-phase peptide synthesis of full-length 72 amino acid one-exon Tat was performed with a sequence derived from the isolate HIV-1_{BRU} on an Applied Biosystems 433A peptide synthesizer by standard Fmoc-Strategy. For synthesis of the acetylated Tat protein (N_ε-Lys50), the N_ε group of K50 was protected by the ivDde group [N_ε-1(4,4 dimethyl-2,6-dioxocyclohex-1-ylidene)-3-methylbutyl]. To deprotect the ivDde group in K50, the peptide was treated with 5% hydrazine in dimethylformamide for 5 min and one part of the product was acetylated with acetic anhydride. Both acetylated and non-acetylated Tat peptides were fully deprotected with trifluoracetic acid, containing 3% triisopropylsilane and 5% water. The peptides were purified to homogeneity by reverse-phase high-pressure liquid chromatography. The correct molecular weights of 8340 Da for the Tat and 8382 Da for the acetylated Tat were established by positive-ion ESI mass spectra recorded on an ion trap Finnigan LCQ mass spectrometer.

Antibody generation and use

Recombinant CBP and PCAF bromodomain proteins were generated as previously reported (Dhalluin *et al.*, 1999). PCAF bromodomain protein was conjugated to activated keyhole limpet hemocyanin (Pierce). CHB rabbits were immunized subcutaneously with 150 μg of conjugated protein in complete Freund's adjuvant (Sigma) followed by four boosts in incomplete Freund's adjuvant. Immunoglobulin G (IgG) fractions were purified with Gammabind plus Sepharose (Amersham Pharmacia Biotech). For ELISA, recombinant PCAF BD or CBP BD proteins were coated overnight at 4°C on Maxisorp polystyrene plates (Nalge Nunc International) and blocked with milk (5% skimmed dry milk in T-TBS). After incubation with anti-PCAF BD IgGs at the indicated concentrations, reactions were developed with 10 μg/ml anti-rabbit IgG-HRP (Jackson ImmunoResearch Laboratories) and 5 mg of orthophenylenediamine dihydrochloride (OPD)/ml H_2O (Sigma) as a colorimetric reagent. Reactions were stopped after 30 min, and absorbances read at 492 and 620 nm on a Multiskan MS ELISA plate reader (Labsystems). For western blot analysis, 50 μg of total cell lysate of HEK 293 cells transfected with HA-PCAF or the vector control were analyzed by SDS–PAGE and developed with anti-PCAF BD antiserum (1:100) or anti-HA monoclonal antibody (Roche).

Microinjection experiments

Subconfluent HeLa-Tat cells (70%) were grown on Cellocate coverslips (Eppendorf) and microinjected at room temperature with an automated injection system (Carl Zeiss). Samples were prepared as a 20 μl injection mix containing the LTR-luciferase (100 ng/ml) and CMV-GFP

(50 ng/ml) constructs together with 5 mg/ml anti-PCAF BD IgGs or pre-immune IgGs. Live cells were examined on a Zeiss Axiovert microscope to determine the number of GFP-positive cells. Four hours after injection, cells were washed in cold phosphate buffer and processed for luciferase assays (Promega).

ELISA

Microwell plates were coated with the PCAF bromodomain (1 μg/ml in 10 mM Na or K phosphate, pH 7.0) for 2 h at room temperature. Non-specific binding sites were saturated with a 10% bovine serum albumin (BSA) solution in binding buffer (100 mM Na or K phosphate, 100 mM NaCl, 5 mM dithiothreitol) for 2 h at room temperature. Wells were washed twice with binding buffer containing 0.1% BSA and 0.05% Tween-20 (washing buffer). Increasing concentrations of the Tat peptide (biotin-GGLGISYGRK50KRRQRRRP, acetylated on K50 or not) was allowed to bind overnight at 4°C. Samples were washed four times with washing buffer. Bound peptide was revealed by incubating with a 0.1 μg/ml solution of streptavidin-conjugated HRP for 1 h at room temperature, followed by washes and incubation with tetramethyl benzidine (TMB) as an HRP substrate (Pierce). The reaction was stopped before saturation of the colorimetric reaction by adding 2 M H_2SO_4. The absorbance of the colored product was measured at 450 nm. Absorbance in each well was corrected for the blank obtained in a corresponding well subjected to the complete procedure but containing no PCAF BD. Results are the average of duplicate samples in one representative experiment.

Tat–PCAF interaction in vitro

Purified GST–PCAF bromodomain proteins (1 μg) were incubated with full-length synthetic acetylated or non-acetylated Tat proteins (5 μg) in binding buffer (97.6 mM NaH_2PO_4, 12.4 mM Na_2HPO_4, 250 mM NaCl, 30 mM Na pyrophosphate, 5 mM EDTA, 10 mM NaF, 0.1% NP-40) for 10 min at 30°C. Precleared and blocked (3% BSA) glutathione beads (Pharmacia) were added to the above mixture for another 30 min at 4°C, washed extensively and resuspended in Laemmli buffer. Binding of Tat to the GST–PCAF bromodomain was analyzed by silver staining or by western blot with a guinea pig anti-Tat antibody.

Tat-TAR binding in vitro

Streptavidin–Sepharose beads (Amersham Pharmacia Biotech, Uppsala, Sweden) were blocked for 1 h at 4°C in binding buffer containing 3% nuclease-free BSA (Amersham Pharmacia Biotech). After equilibration in binding buffer, 30 μl beads were incubated with 0.5 μg biotinylated TAR element (biotin-AATTCCAGATCTGAGCCTGGGAGCTCTCT-GGA; Xeragon, Zurich, Switzerland) and incubated for 1 h at 4°C. TAR bound to beads was mixed with 2 μl lysates from Tat expression plasmids *in vitro* translated in the presence of 20 μCi [^{35}S]methionine (Amersham Pharmacia Biotech) using the TNT T7 coupled reticulocyte Lysate System (Promega, Madison, WI). Reactions were incubated for 10 min at 30°C and twice washed with binding buffer prior the addition of loading buffer. Proteins were separated by SDS–PAGE, fixed and amplified (Amplify; Amersham Pharmacia Biotech) for 30 min, dried and exposed to a BioMax MR film (Kodak, Rochester, NY).

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The Histone Acetyltransferase, hGCN5, Interacts with and Acetylates the HIV Transactivator, Tat*

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Factor acetyltransferase activity associated with several histone acetyltransferases plays a key role in the control of transcription. Here we report that hGCN5, a well known histone acetyltransferase, specifically interacts with and acetylates the human immunodeficiency virus type 1 (HIV-1) transactivator protein, Tat. The interaction between Tat and hGCN5 is direct and involves the acetyltransferase and the bromodomain regions of hGCN5, as well as a limited region of Tat encompassing the cysteine-rich domain of the protein. Tat lysines 50 and 51, target of acetylation by p300/CBP, were also found to be acetylated by hGCN5. The acetylation of these two lysines by p300/CBP has been recently shown to stimulate Tat transcriptional activity and accordingly, we have found that hGCN5 can considerably enhance Tat-dependent transcription of the HIV-1 long terminal repeat. These data highlight the importance of the acetylation of lysines 50 and 51 in the function of Tat, since different histone acetyltransferases involved in distinct signaling pathways, GCN5 and p300/CBP, converge to acetylate Tat on the same site.

Histone acetyltransferases (HATs)[1] are enzymes capable of acetylating specific lysine residues in the N-terminal tails of core histones. This acetylation would modify DNA-nucleosome or nucleosome-nucleosome interactions, and then facilitate gene activation (reviewed in Refs. 1–4). The link between histone acetylation and transcriptional activation has been confirmed by the detection of a HAT activity associated with transcription factors, some characterized first as cofactors of transcriptional activators (reviewed in Ref. 5). Furthermore, it has been shown that histone acetyltransferases p300, CBP, and P/CAF also possess a factor acetyltransferase (FAT) activity, in that they acetylate non-histone substrates, such as specific and general transcription factors or chromatin-related proteins (reviewed in Ref. 5). Factor acetylation can modify protein-DNA or protein-protein interaction depending on the nature of the substrate. For instance, acetylation of the transcriptional activators GATA-1 by p300 (6), p53 by p300 and P/CAF (7–9), and E2F (10, 11), or TAL1 (12) by P/CAF increases their DNA-binding capacities, whereas acetylation of E1A 12S and TAL1 by P/CAF inhibits their interaction with a co-repressor (12, 13). Finally, the acetylation of the chromatin-associated protein HMG-17 by P/CAF reduces its affinity for nucleosomes (14). Protein acetylation may also regulate other cellular functions such as the stability or nuclear import of proteins (10, 15).

Tat, a viral protein encoded by human immunodeficiency virus 1 (HIV-1), activates viral gene transcription from the proviral long terminal repeat (LTR) by interacting with several cellular factors. One such factor is the Tat-associated kinase CDK9/P-TEFb, which is recruited by Tat on the transcription response RNA (TAR) element, located at the 5′ termini of all viral transcripts. This complex stimulates transcriptional elongation by phosphorylating RNA polymerase II C-terminal domain (Refs. 16 and 17; reviewed in Refs. 18 and 19).

Interestingly, it has recently been shown that in addition to cellular kinases Tat can also recruit cellular HATs (20–24). Two nuclear HATs, p300 and P/CAF, were found to interact with and acetylate Tat on distinct lysine residues (25, 26). The acetylation of the activator domain of Tat by P/CAF enhances the binding of Tat to the cellular factor CDK9/P-TEFb, whereas the acetylation of the TAR binding domain of Tat by p300 promotes its dissociation from TAR element during early transcriptional elongation, and both events increase the activation of transcription from the LTR. Thus, HIV-1 appears to develop the capacity to use the cellular acetylation signaling system to enhance its transcription (25, 26) and probably to control various cellular functions (20, 24).

GCN5 is one of a number of well characterized nuclear HATs that acetylate histones H3 and H4 at specific residues (27, 28). GCN5 is a conserved protein from yeasts to humans (27, 29–31). Human GCN5 (hGCN5) exists in two forms resulting from alternative splicing (30, 31). The longer form (813 amino acids) shares strong homologies with P/CAF. The shorter form (476 amino acids) exhibits a size similar to yeast GCN5 and corresponds to the C-terminal part of the long hGCN5.

In contrast to its homologue P/CAF, GCN5 has been described as a poor transcriptional coactivator in mammals. Additionally, it is not known whether hGCN5, like p300, CBP, and P/CAF, uses its acetyltransferase activity to modulate transcription factors by acetylation.

In this report we investigated the ability of hGCN5 to acetylate and modulate the activity of the HIV transactivator, Tat. Data presented here show that hGCN5 interacts directly with Tat. The domains involved in this interaction were mapped on both proteins and the site of acetylation on Tat characterized. hGCN5 was found to acetylate Tat *in vitro* on lysine 50 and 51.

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[1] The abbreviations used are: HAT, histone acetyltransferase; FAT, factor acetyltransferase; HIV, human immunodeficiency virus; GST, glutathione *S*-transferase; TAR, transcription response RNA; LTR, long terminal repeat; CAT, chloramphenicol acetyltransferase; wt, wild type; PCR, polymerase chain reaction.

In agreement with these findings, hGCN5 was shown to stimulate Tat-dependent transcriptionnal activation of the HIV-1 LTR. These results point to hGCN5 as a novel "FAT" that can take part in the activation of HIV gene expression by acetylating the viral transactivator Tat.

MATERIALS AND METHODS

Plasmid Constructs—Expression vectors for short hGCN5, wild type (wt), and mutants were constructed as follows. Coding sequences for hGCN5 and different mutants were produced by PCR with appropriate primers. Forward primers are: 5′-GCTCTAGACATGCTGGAGGAGGAGATC for wt and mutants 1–388 and 1–110, 5′-GCTCTAGACATGCTGGGGCCTGAGAC for mutants 111–476 and 111–251, 5′-CCTCTAGACTACGCCGACGAGTACGCC for mutants 252–476 and 252–388, and 5′-CCTCTAGATGCCTGGCCCTTCATGGAG for mutant 389–476. Reverse primers are 5′- GGCGGATCCCTACTTGTCAATGAGGCCTCC for wt and mutants 111–476, 252–476, and 389–476; 5′- CGGGATCCTAGGCAGCAGGGTCAGTGA for mutant 1–110; 5′-TCGGATCCTAGGTGAGGAAGTAGAGAA for mutant 111–251; 5′- CGGGATCCTAACTGGGGTGAGACTTGATTT for mutants 1–388 and 252–388. Forward and reverse primers contained *Xba*I and *Bam*HI restriction sites, respectively. PCR products were cloned in pSG5 (Stratagene).

The expression vector for GST-Tat M5 mutant was generated as follows. The amino acids 21–48 region of Tat was amplified by PCR using the following sequences as primers: 5′-GGGGATCCCAACTGCTTGTACCAATTGCT-3′ and 5′-GGAATTCTAGCCATAGGAGATGCCTAAG-3′. The amplified fragment was then cloned in *Bam*HI-*Eco*RI sites of pGEX-5X.3 (Amersham Pharmacia Biotech).

Cell Culture and Transfections—HeLa cells and HL3T1 cells (National Institutes of Health AIDS research), grown in monolayers to 50% confluence, were transfected by the calcium phosphate co-precipitation method with variable amounts of different plasmids (as described in the legends of the figures). Total amounts of SV40 and cytomegalovirus promoter-containing plasmids were adjusted with pSG5 and a pCMV vector DNA. Cells were collected 36 h after transfection and used for gene reporter assay. In each experiment, the expression of the transfected proteins (wild type and mutants Tat as well as hGCN5) was controlled by a Western blot using anti-Tat or anti-flag antibodies.

Reporter Gene Assays—The luciferase activities was measured on HeLa cytoplasmic extracts using a luciferase-based assay system (Promega), and the level of CAT protein was measured on HL3T1 extracts using an enzyme-linked immunosorbent assay (Roche Molecular Biochemicals), according to the manufacturer's instructions. The luciferase activity was normalized with respect to the plasmid uptake. Briefly, nuclei and cell debris obtained after the luciferase assay were pelleted and resuspended in 100 μl of 10 mM Tris-HCl pH 8, 1 mM EDTA containing 0.5% SDS, and digested with 0.1 mg/ml proteinase K. Ten μl of each sample were used to perform a Southern blot analysis. The blot was probed with a ^{32}P-labeled HIV-1 LTR fragment. Signals were quantified using a PhosphorImager, and the obtained values were used to normalize the luciferase activity.

Production of Recombinant hGCN5—A recombinant His-tagged hGCN5 was produced in BL21-Lys bacteria transformed with the appropriate expression vector (pET28a-hGCN5). Briefly: recombinant protein expression was induced in exponentially growing bacteria by 0.5 mM isopropyl-1-thio-β-D-galactopyranoside, overnight at room temperature. Bacteria were then pelleted, resuspended in 10 ml of lysis buffer (50 mM $NaPO_4$, 300 mM NaCl, 0.5% Tween 20, 10% glycerol, 15 mM β-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride) containing 10 mM imidazole (per 500 ml of cell culture), and lysed by sonication. After centrifugation, supernatant was loaded on a Ni^{2+}-agarose column. The column was washed with lysis buffer containing 20 mM imidazole, and His-tagged hGCN5 was eluted by lysis buffer containing 250 mM imidazole, following by dialysis on 25 mM Tris-HCl, pH 8, 10% glycerol, 100 mM NaCl, 0.1 mM EDTA. Total amounts of protein were estimated on a Coomassie-stained polyacrylamide gel.

GST Pull-down Assays—GST and GST-Tat fusion proteins were produced in *Escherichia coli* BL21 strain as described previously (32) and retained on glutathione-Sepharose beads. Beads were incubated (1 h, 4 °C) either with 300 μg of HeLa nuclear extract (prepared according to the Dignam method) prepared from cells transfected with 1 μg of flag-tagged hGCN5 expression vector, or with 100 μl of a buffer (20% glycerol, 3 mM $MgCl_2$, 50 mM Hepes pH 7.9, 250 mM KCl, 0.1% Nonidet P-40, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 10 μM acetyl-coA) containing 5 μg of purified, recombinant GCN5 or 10 μl of [^{35}S]methionine-labeled hGCN5, wild type, or mutant proteins (produced using the TNT coupled reticulocyte lysate system; Promega). After three washes in the same buffer, complexes were recovered in gel loading buffer and resolved by polyacrylamide gel electrophoresis. The proteins were then revealed either by Western blot using an anti-flag M2 (Sigma) or anti-His antibody (Qiagen), or by autoradiography.

In Vitro Acetylation—Purified recombinant His-GCN5 (2 μg) was incubated (30 min, 30 °C) with purified GST-Tat in a 30-μl reaction buffer (25 mM Tris-HCl, pH 8, 10% glycerol, 100 mM NaCl 0.1 mM EDTA, 0.2 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol, and 0.1 μCi of [^{14}C]acetyl-coA). For assays with Tat mutants, acetylation was performed in same conditions, except that GST-Tat wild type or mutant proteins were immobilized on 25 μl of glutathione-Sepharose beads. Proteins were denatured in gel loading buffer and resolved by polyacrylamide gel electrophoresis. Gels were fixed, stained in Coomassie Brillant Blue, and exposed to x-ray films at −80 °C.

RESULTS

Tat and GCN5 Interact Directly in Vitro—GST-Tat pull-down assays were set up to investigate the ability of Tat to interact with hGCN5: GST-Tat (Tat86, HXB2 strain) fusion protein and GST (as a control) were produced in *E. coli*, immobilized on glutathione-Sepharose beads, and incubated with HeLa nuclear extracts expressing flag-tagged hGCN5 (*f-hGCN5*; Fig. 1*A*). A Western blot using an anti-flag antibody revealed that flag-tagged hGCN5 is efficiently retained on GST-Tat beads, but not on GST. hGCN5 present in the HeLa nuclear extracts could therefore specifically interact with Tat.

To show direct interaction between hGCN5 and Tat, GST-Tat pull-down assays were performed with purified recombinant His-tagged hGCN5 protein, followed by Western blotting using an anti-His antibody for detection of bound tagged hGCN5. Tat from two different viral strains, Tat 86 (HXB2 strain) and Tat 101 (SF2), were produced in fusion with GST. Fig. 1 (*B* and *C*) shows that recombinant hGCN5 is specifically retained on Tat 86 as well as on Tat 101. We can conclude from these experiments that hGCN5 is capable of interacting directly with both forms of Tat, without any cellular intermediates.

Mapping the Tat-interacting Domains of GCN5—To determine the domains of hGCN5 involved in interaction with Tat, we constructed several hGCN5 deletion mutants, corresponding to regions encompassing amino acids 1–110, 111–251 (contains the HAT domain), 252–388, and 389–476 (contains the bromodomain), or combinations of two or three adjacent domains (Fig. 2*A*). ^{35}S-Labeled mutants were generated *in vitro* and tested for their interaction with Tat in a GST-Tat pull-down assay. The N-terminal 1–110 part of hGCN5 as well as the 252–388 region were unable to interact with Tat (Fig. 2, *B* (*lanes 2* and *6*) and *D*). Two regions of hGCN5, encompassing amino acids 111–151 and 389–476 (corresponding to HAT and bromodomain, respectively) efficiently interacted with Tat (Fig. 2, *B* (*lanes 4* and *8*) and *D*). All the hGCN5 fragments used in the experiment presented on Fig. 2 (*C* and *E*) contained either the HAT or the bromodomain and consequently interacted with Tat.

We conclude from these results that the HAT domain and the bromodomain of hGCN5 are two Tat-interacting modules and that the interaction of Tat with one domain may occur independently of the interaction with the other domain.

Mapping hGCN5-interacting Domain of Tat—We then mapped Tat domains necessary for hGCN5 interaction. GST-Tat pull-down assays were carried out with different C-terminal truncated forms of Tat 101 (Fig. 3*A*) fused to GST, immobilized on glutathione-Sepharose beads, and incubated with purified recombinant hGCN5 as described. Results show that the hGCN5-Tat interaction is maintained for a Tat fragment containing the 48 first residues (Fig. 3*B*, *M3 mutant*, *lane 5*). However, the interaction is completely abolished for the M4 mutant (amino acids 1–22) (Fig. 3*B*, *lane 6*). These results suggested that the Tat domain responsible for inter-

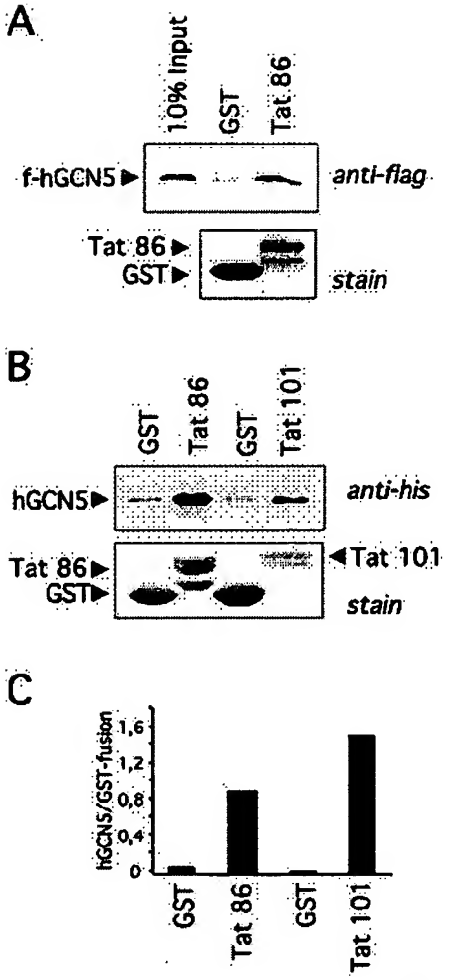

FIG. 1. **Direct interaction between Tat and hGCN5.** *A* and *B*, GST or GST-Tat were immobilized on glutathione-Sepharose beads and incubated either with a nuclear extract from flag-hGCN5-expressing HeLa cells (*f-hGCN5*) (*A*) or with purified recombinant His-tagged hGCN5 (*B*). After washes eluted complexes were loaded on a 10% polyacrylamide gel, and the retention of hGCN5 on GST or GST-Tat was analyzed by a Western blot using anti-flag (*A*) or anti-His (*B*) antibodies. The quality and amounts of the purified GST and GST-Tat used was analyzed by loading a fraction of eluted complexes on a 12% polyacrylamide gel and by Coomassie Blue staining of the gel (*stain panels*). *C*, the film and the gel shown in *B* were scanned to quantify Tat-hGCN5 interactions. The values corresponding to the intensities of the bands of interest were calculated using NIH Image software (rsb.info.nih.gov/nih-image/). Histograms show the ratio of the values obtained for hGCN5 to that of the bands on the stained gel (GST fusion).

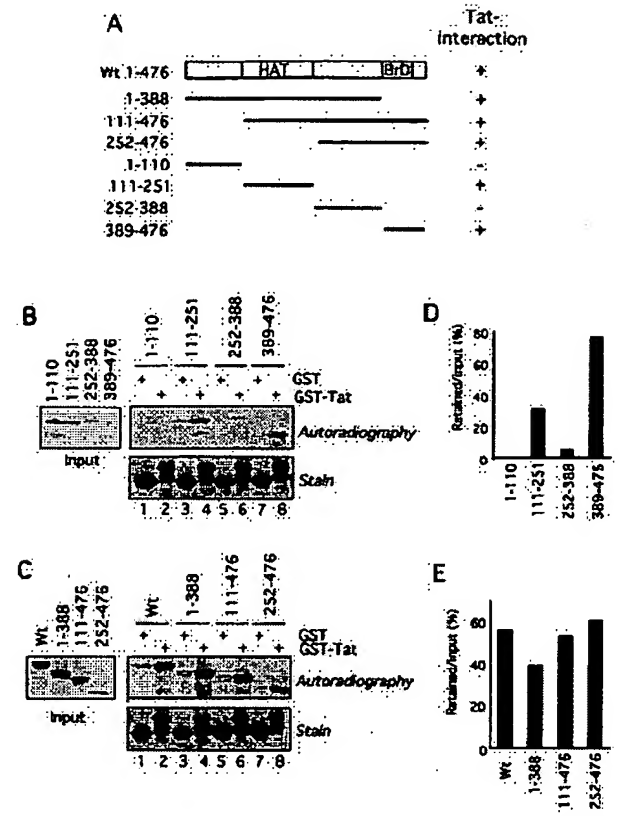

FIG. 2. **Tat interacts with HAT and bromodomain of hGCN5.** *A*, schematic representation of hGCN5 wild type (*Wt*) and deletion mutants. *B* and *C*, wild type and mutant forms of ^{35}S-labeled hGCN5 were incubated with immobilized GST or GST-Tat. After washes and denaturation of complexes, proteins were loaded on 12% (*B*) or 10% (*C*) polyacrylamide gel. Coomassie staining of gels served to control the quality and the quantity of GST and GST-Tat (*Stain panels*). Retention of hGCN5 mutants on GST-Tat was analyzed by autoradiography (*Autoradiography panels*) and compared with 10% of total input (*Input panels*). *D* and *E*, Tat-hGCN5 interactions were quantified as in Fig. 1*C* except that histograms indicate the ratio of retained to input hGCN5 fragments (expressed as percentage).

acting with hGCN5 was localized between amino acids 22 and 48. In order to confirm this hypothesis, we generated a new Tat mutant containing only the amino acids 21–48 region of the protein fused to GST (M5 mutant). Interestingly, hGCN5 interacted with this Tat mutant as efficiently as with the wild type protein (Fig. 3*B*, *lanes 8* and *9*). This experiment suggests that the amino acids 21–48 region of Tat is necessary and sufficient for the interaction of Tat-hGCN5. This region contains the cysteine-rich and core domains of Tat and is located in the minimal activation domain of the protein.

hGCN5 Acetylates Tat on Residues K50 and K51—Recently, it has been discovered that p300 and P/CAF acetyltransferases modulate Tat transcriptional activity by directly acetylating the protein on specific and distinct lysines (25, 26). We examined if hGCN5 was also capable of acetylating Tat. Purified GST-Tat or GST (as a negative control) was incubated with increasing amounts of purified recombinant hGCN5, in the presence of ^{14}C-labeled acetyl-CoA. Proteins were then electrophoresed on a denaturing gel, and the acetylation of Tat revealed by autoradiography. We observed that the incubation of Tat with increasing amounts of hGCN5 led to the appearance of two major radiolabeled bands corresponding to GST-Tat and hGCN5, respectively (Fig. 4*A*, *right panel*). This experiment also showed that, in addition to acetylating Tat, hGCN5 is also capable of undergoing auto-acetylation. In the control experiment, using GST as a substrate, only auto-acetylation of hGCN5 was observed (Fig. 4*A*, *left panel*). We concluded from these experiments that hGCN5 specifically acetylates Tat *in vitro*.

To define which lysines of Tat are the site of acetylation by hGCN5, similar experiments as above were performed using the wild type Tat 101 or Tat deletion mutants as substrate (Fig. 4*B*). hGCN5 could efficiently acetylate wild type Tat, and M1 and M2 mutants, but could not acetylate Tat M3 and M4 mutants (Fig. 4*C*, *lanes 5* and *6*). The region of Tat acetylated by hGCN5 is therefore located between amino acids 48 and 60 and corresponds to the basic domain of Tat. This region contains only two lysines at positions 50 and 51 (Fig. 4*B*). Point mutations of either Lys-50 or Lys-51 were then generated to determine which lysine was the target of hGCN5. In both cases, mutation of a single lysine reduced the acetylation of Tat (Fig.

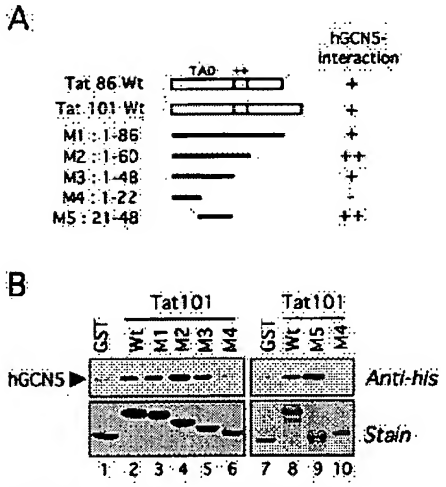

FIG. 3. **hGCN5-interacting domains of Tat.** *A*, schematic representation of Tat wild type (*Wt*) and deletion mutants. Tat is composed of a minimal transactivating domain (*TAD*) (amino acids 1–48), an RNA binding domain (amino acids 49–57) rich in positively charged basic residues (++), and a C-terminal domain. *B*, Tat wild type or indicated mutants fused to GST and immobilized on glutathione-Sepharose were incubated with purified recombinant hGCN5 as described in the legend for Fig. 1*B*. Presence of hGCN5 was analyzed by an anti-histidine antibody (*Anti-his panel*). A Coomassie-stained polyacrylamide gel shows the quantity and the quality of GST-Tat mutants used (*Stain panel*).

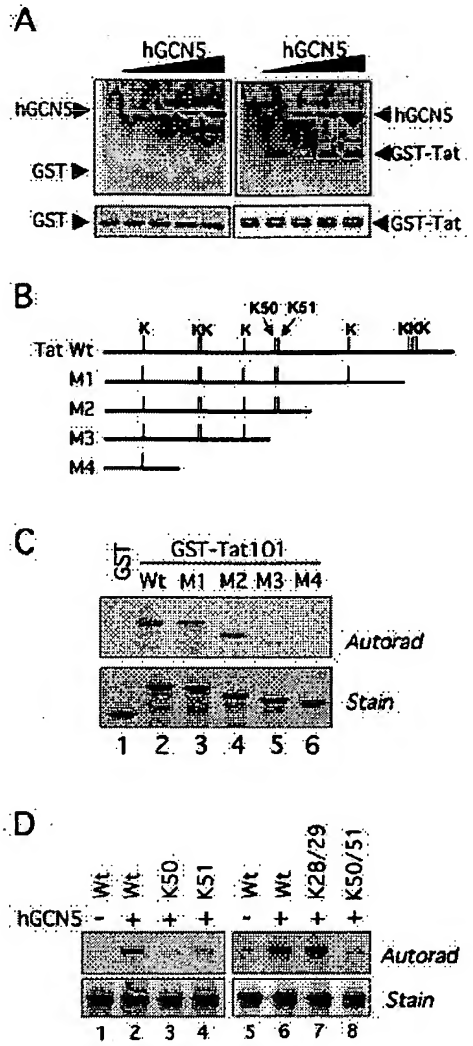

FIG. 4. **hGCN5 acetylates Tat on lysines 50 and 51.** *A*, GST (*left*) or GST-Tat 86 (*right*) were purified and incubated at room temperature with increasing amounts of recombinant hGCN5 in the presence of [^{14}C]acetyl-CoA. Samples were then denatured and loaded on a 12% polyacrylamide gel. Presence of equivalent amounts of GST or GST-Tat was controlled by Coomassie staining (*lower panel*), and acetylation of proteins was revealed by autoradiography of the gel (*upper panel*). *B*, schematic representation of the localization of lysines (*K*) on Tat wild type (*Wt*) or mutated (*M1*, *M2*, *M3*, and *M4*). The position of lysines 50 and 51 is indicated. *C*, Tat 101 deletion mutants fused to GST and immobilized on glutathione-Sepharose were incubated at room temperature with recombinant hGCN5 in the presence of [^{14}C]acetyl-CoA. Analysis of Tat acetylation was performed as in *A*. *D*, Tat 101 point mutants fused to GST were immobilized on glutathione-Sepharose and incubated with [^{14}C]acetyl-CoA in the presence (+) or absence (−) of recombinant hGCN5, as indicated. Analysis of Tat acetylation was performed as in *A*. *K50*, *K51*, *K28/29*, and *K50/51* represent the replacement of the indicated lysines by arginines.

4*D*, compare *lanes 3* and *4* to *lane 2*). This result suggested that both lysines were acetylated by hGCN5. In order to confirm this hypothesis, Tat containing Lys-50 and Lys-51 mutated to arginines was generated. As controls, we also mutated lysines at position 28 and 29 to arginines. These mutants were tested as substrates for acetylation by hGCN5 (Fig. 4*D*, *lanes 7* and *8*). As expected, the replacement by arginines of both Lys-50 and Lys-51, but not that of Lys-28 and Lys-29, reduced the acetylation of Tat by hGCN5 to the background level (Tat acetylation in the absence of hGCN5, *lane 5*). Thus, both lysines 50 and 51 of the HIV-1 transactivator Tat are targets for hGCN5-mediated acetylation.

Cooperation of Tat and hGCN5 on HIV LTR—The acetylation of lysines 50 and 51 of Tat has been shown to correlate with an efficient transactivation of the HIV LTR (25, 26). We therefore examined the effect of hGCN5 on Tat-dependent LTR activation. HeLa cells were co-transfected with an LTR-luciferase reporter plasmid, and expression vectors for Tat, either wt or Tat mutated on lysines 50 and 51 (lysine to arginine mutation), in the presence or absence of hGCN5 (Fig. 5*A*). As expected, wild type Tat strongly activated the HIV LTR (about 40-fold in our assay). In contrast, hGCN5 alone only poorly activated the LTR. Interestingly, we found that the co-expression of Tat and hGCN5 considerably enhanced the transactivator potential of Tat. Under the same conditions, Tat Lys-50/Lys-51 mutant, although capable of efficiently stimulating the HIV-1 LTR transcriptional activity, was unable to cooperate with hGCN5 to further activate transcription.

In order to know if the stimulating effect of hGCN5 on the HIV promoter could be observed on an integrated, chromatin-associated LTR, similar experiments were performed in HL3T1 cells, a HeLa cell-derived line containing an integrated LTR-CAT construct (Fig. 5*B*). In the absence of Tat, the integrated LTR exhibited no detectable transcriptional activity (data not shown). In contrast, in cells transfected with 10 ng of the wild type Tat expression vector, the LTR was strongly activated. This Tat-dependent transcriptional activity was further enhanced, although to a lesser extent than that of the non-integrated LTR, by its coexpression with hGCN5 (Fig. 5*B*), Here again, although Tat Lys-50/Lys-51 mutant efficiently stimulated the HIV-1 LTR transcription, it was not as efficient as the wild type protein in cooperating with hGCN5.

These results strongly suggest that acetylation of Tat on Lys-50 and Lys-51 by hGCN5 plays an important role in the control of the activity of the protein.

DISCUSSION

The HIV transactivator Tat interacts with several histone acetyltransferases, such as Tip60 (24, 33), hTAF$_{II}$250 (20), p300, CBP, and P/CAF (21–23). We report here that hGCN5 is a novel Tat-interacting HAT. This interaction occurs directly,

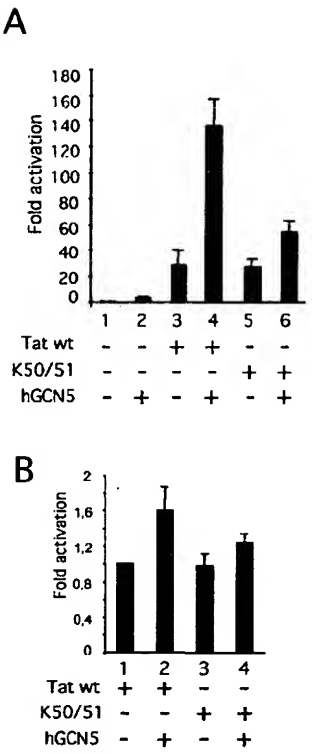

FIG. 5. **hGCN5 enhances the activation of the HIV LTR by Tat.** *A*, HeLa cells were co-transfected with 50 ng of reporter plasmid pLTR-Luc (luciferase gene under LTR control), 0 or 10 ng of the Tat (wt or Lys-50/Lys-51 (*K50/51*) double mutant) expression vector pSGTat, and 0 (−) or 400 ng (+) of the flag-tagged hGCN5 expression vector pf-GCN5, as indicated. Total amounts of DNA for transfections were maintained constant by addition of empty control vector. Luciferase activity was measured with a luminometer and normalized with respect to the plasmid uptake as indicated under "Materials and Methods." Mean values of at least three independent assays are represented. *B*, HL3T1 cells, containing an integrated LTR-CAT reporter gene, were co-transfected with 10 ng of pSGTat wild type or K50/51 mutant and 0 (−) or 400 ng (+) of pf-GCN5. Transfections were performed as described in *A*. Quantification of the CAT protein was carried out by an enzyme-linked immunosorbent assay and mean values of three independent experiments were represented as in *A*.

via residues 21–48 of Tat, a region contained in the minimal transactivating domain of Tat. This finding is in agreement with the fact that the amino acid 30–45 region of Tat was required for recruitment of HAT activity from nuclear cell extracts (21). Interestingly, the Tat hGCN5-interacting domain appears to differ from domains involved in interaction with other HATs. For instance, the basic domain of Tat has been shown to be required for Tip60 and p300 interaction, whereas the Tat C-terminal domain is necessary for $hTAF_{II}250$ interaction (20, 22–24). Otherwise, similar to p300/CBP and P/CAF, hGCN5 is capable of stimulating Tat-dependent LTR transcriptional activation and of acetylating Tat on specific lysines. Tat-Tip60 and Tat-$TAF_{II}250$ interactions do not, however, affect transcription from the LTR but repress transcription of cellular genes such as the manganese superoxide dismutase gene (24) and the major histocompatibility class I genes (20). Thus, although the targeting of HATs would be a general mechanism of Tat activity, the HAT-interacting domain of Tat and possibly the functional consequences of these interactions could be different for each HAT.

In contrast to P/CAF or p300, only a few reports show a direct recruitment of GCN5 by transcriptional factors in mammals. Long hGCN5 is known to be recruited by c-Myc to activate transcription, but this recruitment was shown to be indirect, and to require the cofactor TRRAP (34). A direct interaction has, however, been described between short hGCN5 and the ubiquitous transcription factor, NF-Y. The interaction was mapped to the N-terminal domain of hGCN5 and to the histone-like DNA binding domain of NF-Y (35). Therefore, the recruitment of hGCN5 by Tat, reported here, constitutes a new example of recruitment of hGCN5 by a transcription factor.

Acetylation of non-histone substrates by GCN5 has also been, to our knowledge, poorly described. Acetylation of E1A by yGCN5 and c-Myb by hGCN5 has been recently reported, but the effect of GCN5 on these transcription factors was not further characterized (13, 36). In this report, we have identified Tat as a new substrate of GCN5 FAT activity. It is therefore very probable that GCN5 (like p300 and P/CAF), acetylates different transcription factors, and more generally other cellular and viral proteins (not necessarily involved in transcription) to modulate their activity.

Since c-Myb and Tat are acetylated *in vitro* by GCN5 and P/CAF, and since both HATs are closely related, it can be speculated that hGCN5 and P/CAF have redundant FAT functions. However, our data show divergences between P/CAF and hGCN5 activity on Tat. First, unlike hGCN5, P/CAF does not stimulate Tat transcriptional activity by itself; instead, it enhances the stimulating effect of p300. Indeed, it has been suggested that Tat, p300/CBP, and P/CAF associate in a ternary complex, and that in this complex, p300-P/CAF interaction is required for a stimulating effect of p300 on Tat activity (21). In our experiments we used the short form of hGCN5, which is unable to interact with p300/CBP (37). Therefore, the short hGCN5 would then act on Tat by a different and more direct mechanism, which does not require the simultaneous recruitment of p300. Finally, functional differences between hGCN5 and P/CAF are confirmed by the fact that they target different lysines within Tat; P/CAF acetylates Tat on lysine 28 (25), whereas hGCN5 targets lysine 50 and 51. This observation suggests that, despite strong homology and preferential acetylation of the same lysine (Lys-14) within the same histone (H3) (28, 38), these HATs could in certain cases recognize different lysines in the same substrate.

Our data show that, although divergent in sequence and belonging to different families, hGCN5 and p300/CBP acetylate Tat on the same lysines. The use of different cellular HATs by Tat to acetylate the same site highlights the importance of this post-translational modification for the activity of Tat. In support of this hypothesis, the acetylation of lysine 50 has been correlated to a lower affinity of Tat for the TAR sequence (25). Thus, the acetylation of Tat would increase the rate of its dissociation from TAR element. The consequence of this acetylation would be a faster recycling of each molecule of Tat, and an increased transcription of the HIV LTR. Data presented here show that hGCN5 is also involved in the acetylation-dependent control of Tat activity. However, the role of hGCN5 and other HATs capable of acetylating Tat in activating the LTR transcription in the context of HIV-infected cells remains to be determined.

Interestingly, the hGCN5-interacting domain of Tat (amino acids 21–48) is different from the acetylated region. Moreover, the recruitment of hGCN5 can occur independently of Tat acetylation, as showed by the M3 Tat mutant, which, although not acetylated by hGCN5 (Fig. 4*C*), interacted with this HAT (Fig. 3*B*). Thus, besides the control of Tat acetylation state, the hGCN5-Tat interaction may also target hGCN5 to the HIV-1 LTR to acetylate histones and then displace nucleosomes at the initiation or during the elongation steps of the transcription. Therefore, one may separate the role of Tat-hGCN5 interaction

on HIV-1 LTR chromatin remodeling from its role on Tat acetylation.

Our data show also that the bromodomain of hGCN5 is capable of efficiently interacting with Tat. Bromodomains are conserved sequence motifs probably involved in protein-protein interactions (reviewed in Refs. 39 and 40). This domain is found in transcription-related proteins and especially in the Tat-interacting HATs p300/CBP, P/CAF, and $TAF_{II}250$ (besides hGCN5). Recently, it has been shown that bromodomains of P/CAF, $TAF_{II}250$, and GCN5 directly and specifically interact with acetylated histones (41–43). Moreover, in the case of hGCN5, the bromodomain is necessary for nucleosome remodeling by the Swi/Snf complex (44). By contacting the bromodomain of hGCN5, Tat could modify the action of hGCN5 on chromatin. It would therefore be interesting to know if such an interaction could perturb either recruitment of hGCN5 on acetylated histones or the activity of the Swi/Snf complex. Tat-hGCN5 interaction would then have consequences on the transcription of cellular genes. Furthermore, although the involvement of p300, P/CAF, and $hTAF_{II}250$ bromodomains in the direct interaction with Tat has not been demonstrated, the Tat-bromodomain interaction could be a general mechanism of modulation of chromatin targeting by bromodomain-containing proteins.

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